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The main objective of this research project has been to apply the T-body approach for the immunotherapy of breast cancer. To this end we have altered the specificity of patient-derived lymphocytes through stable modification with chimeric receptor genes consisting of either an anti-breast tumor single-chain variable (scFv) region of anti-HER2 antibody or the Erb-B NDF ligand linked to a T cell activation molecule (the γ subunit of the Fcε receptor).Both receptors recognize molecules specifically overexpressed on many breast tumors. A special receptor configuration developed and tested in this study included part of the extracellular and the entire intracellular domains of the co-stimulatory CD28 molecule in-between the scFv and the gamma moieties of the chimeric receptors. This three partied receptor appeared functional in redirecting hybridoma cell lines, human PBLs and resting lymphocytes of transgenic mice. Such genetically modified redirected lymphocytes will be tested on explanted breast tumor cells for specific lysis and secretion of cytokines *in vitro*, and for elimination of human breast tumor cells passaged in NOD/SCID mice *in vivo*. These model systems will serve to determine the optimal conditions towards clinical trials. Altogether, this therapeutic strategy may allow new approaches towards the adoptive immunotherapy of breast cancer in humans.

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INTRODUCTION

In this proposal we suggest a novel approach for the immunotherapy of breast carcinoma. The difficulty of treating metastatic breast cancer with conventional therapy, combined with the presence of defined tumor- or tissue-associated antigens on breast tumors, makes this malignancy an ideal candidate for an immunotherapeutic approach to treatment.

One method developed for the immunotherapy of cancer is to remove lymphocytes from tumors obtained at surgery or biopsy, expand them *ex vivo* in the presence of lymphokines, and reinfuse these cells into the patient. Treatment of cancer by the infusion of autologous tumor infiltrating lymphocytes has produced clinical responses in some patients. Although several explanations can be proposed for the limited response thus far, one possibility is the lack of specificity of the reintroduced lymphocytes. In addition, this technique is limited by the difficulty in obtaining specific tumor-infiltrating lymphocytes for many histologic types of cancer (including breast cancer). In contrast, many monoclonal antibodies have been described that bind tumor-associated antigens shared by tumors of similar histology. These monoclonal antibodies can be attached to a cytotoxin or to an antibody or growth factor to redirect cytotoxic T cells. However, most clinical attempts using such immunotoxins have not fulfilled expectations. Their therapeutic efficacy is restricted to blood borne tumors, primarily because solid tumors are not sufficiently accessible to antibodies.

Cancer patients usually mount a poor -if any- immune response against their own tumors due to poor tumor immunogenicity and an immunosuppressed state common to many cancer patients. Several approaches have been attempted to enable the immune eradication of tumor cells. These include various methodologies to augment, nonspecifically or specifically, the host immune response and treatment with specific anti-tumor antibodies. Non-specific treatments, such as the use of LAK (lymphokine-activated killer) cells, are not effective in all types of cancer. The requirement for the co-injection of large amounts of IL-2 causes severe side effects which often require the cessation of treatment. The use of antibodies in passive immunotherapy is often of limited efficacy, both because of the difficulty in identifying true tumor-specific antigens, poor tumor penetration, and the short half-life of the antibodies.

Our group has pioneered the T body approach, a novel approach for cancer therapy. The "T body" approach has several advantages over traditional immunotherapeutic methods. We have joined these two approaches of adoptive immunotherapy and immunotoxin therapy to genetically engineer an improved 'immunocytolysin', which is an antibody scFv region attached to a cytotoxic T cell signaling molecule. Genes for chimeric T cell receptors have been constructed containing the coding sequence of an antibody-derived scFv directed against a tumor associated antigen, attached to the transmembrane and intracytoplasmic sequences of a T cell signaling molecule. These genes are then transfected into cytotoxic T cells, thereby conferring upon them the ability to specifically recognize and kill tumor cells. The scFvR design we have developed combines antibody recognition and T cell signaling in one continuous protein and has been used to endow murine and human cytotoxic cells with non-MHC-restricted, antibody-derived specificity. This T-body approach combines the advantage of antibody specificity with the homing, tissue penetration, and target cell destruction mediated by T lymphocytes.

Body

Our desired goal has been to use effector lymphocytes directed towards tumors as a treatment for breast cancer, a type of adoptive immunotherapy. New developments in the field have led to a large increase in the capability to test therapeutic strategies in animal models. Redirected adoptive immunotherapy of tumors requires the introduction of a new receptor into T lymphocytes to reprogram their specificity to be directed against tumor cells. This receptor can be a TCR with the appropriate specificity (1, 2) or even more sophisticated a chimeric T cell receptor where the specificity is derived from an antibody recognition of a tumor antigen (3). This latter configuration has the advantage of being MHC independent, thus individual unrestricted. This specificity can even be supplied by a ligand (4, 5). The antibody can be selected for in immunized mice, in the form of monoclonal antibody and its antigen-recognition unit (V-region) is then genetically attached to a TCR signaling module. The genetic modification of T cells has undergone a huge increase in popularity driven by some key advances in the underlying technology. One of these is the ability of retroviruses to transduce efficiently T lymphocytes (6-18). Introduction of a suicide gene can control the effect of the lymphocytes (19, 20). Although great strides have been made in the ability to transduce T lymphocytes which have been activated, increasing emphasis is being put on the transduction of naïve resting T cells.

CONSTRUCTION OF scFvAb-CD28-Fc ε Receptor γ chain CHIMERA

A model for the self/ non-self recognition of the immune response has lead to the two signal model (21, 22). It has long been known that stimulation of the TCR in naïve lymphocytes leads to anergy a state of profound unresponsiveness to antigenic stimulation. It could be that stimulation through a chimeric receptor in naïve lymphocytes could induce anergy (23, 24). On the other hand there may be some appropriate stimulation in vivo that primes transgenic T cells for chimeric TCR activation (25). Only through the concomitant stimulation of a co-stimulatory molecule like CD28 can an effector response be ensured to be mounted. In fact if tumor cells which are able to present antigens are transfected to artificially express B7-1 (CD80), the ligand for CD28, on their surface, they can stimulate the activation of cytolytic T cells and endows upon them the ability to induce an immune response. CD28 is constitutively expressed on the cells surface of resting human T cells as a homodimer of single immunoglobulin superfamily V-like domains (26). CD28 signaling can protect activated T cells from programmed cell death and stabilize IL-2 mRNA (27-31). High affinity interactions can bypass the CD28 requirement (32-35) even in CD8 positive cells (36). CTLA4, a receptor similar to CD28 but with mostly inhibitory activity, also binds to the B7 ligands of CD28 with an even higher affinity. The soluble CTLA4Ig is an antagonist of CD28 binding and can block tumor rejection (37, 38). Human CD28 is functional in mouse cells (39). A functional chimeric receptor has been made by fusing the CD28 cytoplasmic domain to the extracellular and transmembrane regions of CD2 (40). A receptor made by attaching a single chain Fv (scFv) to the CD28 transmembrane and cytoplasmic domains can be activated by the corresponding antigen (41). One strategy to bypass the requirement for CD28 co-stimulation has been to create a single receptor consisting of three segments, the scFv, the CD28 signaling module and a TCR signaling module such as the γ

chain of the Fcε receptor (42, 43). This receptor (Figure 1) has several advantages: Since it has the transmembrane and cytoplasmic regions of CD28 it can provide for co-stimulation and prevention of apoptosis of CD28 (44-46). This tripartite receptor also maintains the hinge region necessary for homodimerization and allow spacing from the membrane which is advantageous in certain instances (47-50).

CD28 is homologous to CTLA4 and as the structure of CTLA4 has been determined, it can be used as a model for the structure of CD28. Alignment of their amino acid sequences to that of a mouse heavy chain reveals that the sequence in CD28 IHV matches the BstE II site sequence in our single chain VTV (51). Therefore the junction was made so that the junction is at a valine which is conserved between the end of the framework 4 beta sheet of the variable region of the antibody (Kabat numbering 111) and a valine which is just after the last beta sheet in CTLA4. Human CD28 was cloned from PBLs and Jurkat cells using the following primers

#7966 BstE II primer for homodimer CD28 5' CCGGTCACCGTGAAAGGGAAACACCTTTGTCC

#7967 reverse 3' primer 5' CGCTCGAGGTGTCAAGATCTATAGGCTGCGAAGTCGCGTGG

In this way the single chain is attached to the CD28 hinge region, transmembrane region, and cytoplasmic region.

At the end of the CD28 coding sequence was attached the γ chain cytoplasmic region from QVR at a BglII site. This deletes CRLKI at the end of the transmembrane or the beginning of the cytoplasmic region of the Fcε receptor γ chain (52) (Figure 2).

TESTING TRIPLE CHIMERA IN CULTURED CELLS

The ability of this receptor to signal was tested first in a T cell hybridoma. A variant of the murine T cell hybridoma MD45 which is lacking TCR expression, MD45.27J, was transfected with DNA encoding scFv-anti-TNP-CD28-γ driven by a Rous Sarcoma virus promoter and carrying the bacterial neo gene to provide for G-418 resistance in transfectants. A transfectant was selected by drug resistance and shown to produce specific RNA (Figure 3a) and surface protein (Figure 3b).

Membrane compartmentalization has been shown to be important for TCR signaling and a co-stimulation requirement has been shown for the microdomain formation (53-57). GPI linked membrane proteins as well as cholesterol and ganglioside GM1, which is reactive which cholera toxin CTx, (58, 59) are found in these microdomains. LAT, Lck, Fyn, CD44, CD59, CD55, CD48, CDw52 and Thy-1 and Ly-6 in mouse are found in these microdomains (60-71). An independent method of insolubilizing the TCR is by attachment of the cytoskeleton. This is differentiated from the microdomain insolubilization by its sensitivity to cytochalasin B (72-74). The relationship of these structures to the protein complex formed on the membrane known as the immunological synapse is not clear (75, 76). A connection has been found by the observation that rafts serve as nucleation points for actin polymerization (71). This model has been summarized (77). However it is grossly similar to the detergent insolubilization found with oncogenic transformation and other receptor activation (78, 79). Other immunological multisubunit receptors also undergo the same phenomenon (80, 81). Western immunoblotting analysis demonstrated scFv-anti-TNP-CD28-γ protein expression (Figure 4). The detergent

insoluble preparation was prepared as described previously (82). Specific protein, detected only as a high molecular weight complex in the detergent-soluble fraction of cell lysates was resolved at the expected size in the detergent-insoluble fraction, suggesting association with detergent-insoluble lipid rafts. In contrast, a chimeric receptor without the CD28 segment was not localized in the insoluble fraction.

The functionality of the chimeric receptor in this hybridoma was checked by examining its response to antigen. The hybridoma was found to produce IL-2 in response to stimulation with either plastic-bound TNP-fowl γ-globulin (FγG) hapten-carrier protein conjugate or with a panel of TNP-modified target cells (Figures 5A and 5B, respectively). The TNP is coupled to FγG at a coupling ratio of 12:1 TNP/FγG for stimulations. Maximal responses to target cells were obtained by stimulation with the A20 B cell line, presumably due to residual antigen-presentation properties. Stimulation with TNP-modified P815 or L1210 transfectants expressing the costimulatory molecule CD28 or the death receptor Fas, respectively, did not significantly modify responses relative to stimulation with the untransfected parental lines.

The cytolytic functions of the hybridoma was tested by determining its effectiveness in killing target cells. It was found to display cytotoxicity towards TNP-modified A20 cells (Figure 5C). Therefore the CD28-based receptor was fully functional in all assays.

INHIBITOR STUDIES

Without the ability to introduce these chimeric receptors into naïve T cells, it is difficult to demonstrate the difference between the chimeric scFv-CD28-γ receptor and the scFv-γ receptor without the CD28 moiety. One possibility to demonstrate the altered drug sensitivity of CD28 compared to CD3 signaling. It was noted early in the study of CD28 that it conferred resistance to cyclosporin A and led to studies on the differential sensitivity of CD28 mediated signaling compared to CD3 mediated signaling (83-85). In lymphocytes, CD28 initiated signaling is cyclosporin resistant. In order to determine whether addition of the CD28 signaling module conferred a differential sensitivity to inhibitors we compared the drug sensitivity of the CD28- γ hybrid to a chimeric receptor containing γ alone. Subclones of a hybridoma transfectant expressing scFv-anti-TNP-CD28-γ were stimulated with TNP-FγG in the presence or absence of pharmacological inhibitors and changes in IL-2 production were monitored (Figure 6). Hybridoma transfectant STG-B expressing scFv-antiTNP-γ was used as a control to confirm CD28-mediated effect. Production of IL-2 triggered through the CD28-γ receptor was found to be specifically resistant to inhibition by the calcineurin inhibitor cyclosporin A as reported for signaling via CD28 versus via TCR/CD3 (39).

CD28 contains an amino acid sequence YMNM which is capable of being phosphorylated and binding phosphatidylinositol 3 kinase. This enzyme is very sensitive to wortmannin. This could be the enzyme which regulates T cell survival through Akt/PKB (86). However CD28 is not the only receptor in the cell which activates PI3K. In addition, hybridomas often show different drug sensitivities than peripheral blood lymphocytes. However our hybridoma did show a differential sensitivity when the CD28 signaling module was present on the chimeric receptor. In the presence of the PI-3 kinase inhibitor wortmannin, IL-2 production triggered via the scFv-CD28-γ receptor was specifically upregulated, as reported for signaling via CD28 versus

via TCR/CD3. It has been reported that at 10 uM wortmannin CD3 dependent IL-2 production is inhibited whereas CD28 IL-2 production is stimulated (87).

GF 109203X, a bisindolylmaleimide, is used as a broad specificity inhibitor of the protein kinase C family of enzymes which are ser/thr phosphorylating enzymes (88). The classical and novel subfamilies are activated by phorbol esters and diacylglycerol, a product of phospholipase C action, whereas the atypical family lacks a DAG binding domain. At a concentration of 30 nM GF109203X has been reported to inhibit stimulation by CD3 antibody + PMA 81%, whereas CD28 antibody +PMA stimulated IL-2 synthesis in Jurkat cells was inhibited 22%, strikingly more resistant (89). In our hybridoma, production of IL-2 triggered through the scFv-CD28-γ receptor was found to be specifically more resistant to inhibition by the PKC inhibitor GF109203X than the scFv-γ receptor, as reported for signaling via CD28 versus via TCR/CD3.

TRANSGENIC MICE

Adoptive transfer of transgenic lymphocytes in a mouse model can be a powerful way to examine cytolytic T cell function in vivo (90-94). We have also introduced this molecule into the mouse germline to create a transgenic mouse for animal models of chimeric T cell receptor therapy. The mouse, and even human, FcεRI γ chain has been introduced into mice lacking the zeta chain and shown to replace it functionally (95, 96). Two different systems were used for gene expression. One was a CD3 delta promoter driven expression vector (97) and the other was driven by a CD2 promoter with a locus control region to ensure expression no matter where the gene is inserted in the genome (98). Locus control regions can overcome heterochromatin induced gene inactivation (99). We then plan to use these transgenic T cells in an adoptive therapy protocol to eradicate tumor cells (100).

In order to test these concepts we created chimeric receptors with the specificity of anti-trinitrophenyl (TNP) or anti-HER2. The anti-TNP specificity can retarget modified T cells to any cell suitably TNPylated thereby providing an excellent test system to the T cell function. HER2 is overexpressed on breast cancer cells and can provide an appropriate target for tumor cells.

A transgenic C57BL/6 mouse was made with about 50 copies of the transgene scFv-antiTNP-γ driven by the CD3 delta promoter. This construct integrated into the Y chromosome (Figure 7) and expressed RNA, particularly well in the thymocytes (Figure 8). When outbred with BALB or SJL expression of mRNA increased as previously reported for a different transgene (101, 102) (Figure 9). Despite the fact that the E. coli gpt gene activated Ssm1 methylation in that transgene (103) and our construct does not contain that sequence, possible some other sequence can activate Ssm in our construct. Although the splenocytes were activated with anti-CD3 antibody, they were not activated by TNP-FγG.

To generalize these experiments to a breast tumor antigen, a chimeric receptor was made with an antibody specificity to HER2. This receptor, scFvN29-Ig-γ, was expressed using the CD3 delta regulatory sequences in MD45/27J cells (Figure 10). Transgenic mice made with this receptor also had the genes integrated into the Y chromosome (Figure 11). This gene was expressed as checked by immunoblotting (Figure 12A) and FACS (Figure 12B). Outbred transgenic mice expressed high levels of RNA (Figure 13A) and had

higher levels of this receptor on their splenocytes (Figure 13), however we were not able show that this receptor is functional.

In order to test the effect of the triple chimera containing both the CD28 and Fcε receptor γ chain, a chimeric receptor scFv-anti-TNP-CD28-γ driven by the CD3 delta regulatory sequences was integrated into the C57Bl/6 mouse genome in 40-60 copies (Figure 14). mRNA for the scFv-anti-TNP-CD28-γ receptor was expressed in the splenocytes of these mice. Immunoprecipitation and immunoblotting analysis also demonstrated expression of transgene protein in splenocytes of these mice (Figure 15A). Immunofluorescence analysis of thymocytes from Tg5.3 mice indicated low levels of transgene surface expression (Figure 15B)).

In an attempt to increase expression a similar construct was expressed with a CD2 regulatory region containing a locus control region (98). However this did not have the desired result (Figure 16).

To test the hypothesis that chimeric receptor can trigger signal transduction pathways capable of activating untreated primary splenocytes, the splenocytes were stimulated with immobilized TNP-FγG and then IL-2 production and proliferation were measured at various time points. Prior to plating of cells, plates were coated with 10 μg/ml FγG or TNP-FγG in phosphate buffered saline (PBS) overnight at 4°C, washed and blocked. Stimulations were performed under two different conditions, either high or low cell density.

High cell density stimulations consisted of plating 20 x 10^6 splenocytes (approximately 12 x 10^6 T cells) in a volume of 4 ml in 6-well plates. Low cell density stimulations consisted of plating 2.5 x 10^5 splenocytes in triplicate in a volume of 200 μl in microtiter plates.

Positive control stimulations were carried out with immobilized 2C11 in microtiter plates coated as described above or with 10 ng/ml TPA plus 1 μM calcium ionophore. The scFv-antiTNP-γ and scFv-antiHER2-γ expressing T cell hybridoma transfectants STG-B and γ1.4, respectively, were used as positive control effectors in IL-2 production assays. Production of IL-2 was assessed in triplicate by CTLL bioassay of 50 μl aliquots of culture supernatants. Proliferation of CTLL was assessed by addition of 50 μl of 25 μM XTT/25 μM phenazine methosulfate (PMS) dissolved in RPMI to cells in a volume of 100 μl. Following addition of XTT/PMS, cultures were incubated at 37°C for 18-20 hours and OD_{450} was measured. The proliferation index is the fold increase in cell number caused by TNP stimulation and is defined as the number of TNP-FγG-stimulated cells divided by the number of FγG-stimulated cells where cell number is obtained by converting OD_{450} values via a standard curve.

Proliferation of splenocytes stimulated at high cell density was assessed in triplicate on 50 μl aliquots of resuspended cell culture. They were processed in the same manner as above except the concentration of XTT was raised to 50 μM.

a) Activation of untreated primary splenocytes of Tg5.3 mice by scFv-antiTNP-CD28-γ

IL-2 production.

In a high density stimulation, specific production of IL-2 in response to stimulation with immobilized TNP-FγG was observed starting at 48 hours post-stimulation, reached a peak at 72 hours and then declined to background level by 120 hours (Fig. 16 , Fig. 18, top left). In comparison, IL-2 production induced by

stimulation of TCR/CD3 with anti-CD3 mAb gave maximal IL-2 production earlier, at 24 hours and declined to background levels earlier (Fig. 19, left). The maximum levels of IL-2 induced via scFv-antiTNP-CD28-γ were 61% of those induced by TCR/CD3 stimulation.

In a low density stimulation, production of IL-2 in response to stimulation with plastic-bound TNP-FγG was induced at 48h post-stimulation and was sustained until the 96h time-point (Fig. 18, top right). Comparison of the kinetics of IL-2 production induced by stimulation of TCR/CD3 by positive control stimulation with 2C11 versus that induced by scFv-antiTNP-CD28-γ indicates that stimulation via TCR/CD3 induces maximal IL-2 production earlier, at 24 hours, which then also declines to background levels earlier, by 72 hours (Fig. 19, right). By the final time-point at 96 hours, levels of secreted IL-2 induced via scFv-antiTNP-CD28-γ had reached 75% of the maximum levels induced by TCR/CD3 stimulation.

Proliferation.

In a high density stimulation, proliferation increased slightly at 24 hours post-stimulation in response to stimulation with immobilized TNP-FγG, as calculated by the proliferation index. The proliferation index then decreased at the 48 hour and 72 hour time-points and then drastically increased to the highest levels measured in the assay on the final time-point at 96 hours (Fig. 18, bottom left). This sudden elevation in proliferation index late in the assay, when the culture conditions had deteriorated and apoptosis appeared widespread and therefore is an increase in survival of the TNP-FγG stimulated cells rather than increased growth. This may reflect CD28-specific pro-survival, anti-apoptotic mechanisms mediated by signaling via the CD28 sub-domain of scFv-antiTNP-CD28γ.

In a low density stimulation, specific proliferation in response to stimulation with plastic-bound TNP-FγG was observed at 24 hours post-stimulation, increased to maximal levels at 72h and then declined slightly by the final time-point of the assay at 96 h (Fig. 18, bottom right).

Results from preliminary experiments.

The proliferation and IL-2 production that were sustained for at least 96 hours in a low cell density stimulation of untreated primary splenocytes with plastic-bound TNP-FγG has also been displayed by splenocytes from one Tg5.3 mouse out of three in a preliminary optimization experiment (Fig. 20A). Shown in these figures are the negative responses of irrelevant Fv control TgA4 splenocytes transgenic for scFv-antiHER2-CD28-γ. Identical proliferative responses were observed for 48 hours post-stimulation by cells from all three mice, however proliferation of cells of mice Tg5.3-381 and -382 then declined to background levels by the final time-point at 96 hours. This is in contrast to the cells of mouse Tg5.3-380 whose proliferation index continued to increase for at least 96 hours, reaching a value of 3, the highest value obtained in these studies. This marked divergence in proliferative response is mirrored in the observation that the cells of mouse Tg5.3-380 sustained IL-2 production for at least 96 hours compared to only 48 hours at low level for cells from the other two mice. Comparative analysis of transgene transcription by RT-PCR indicates that these splenocytes expressed similar levels of scFv-antiTNP-CD28-γ mRNA prior to stimulation (Fig. 20B), therefore, it appears unlikely that differences in basal levels of transgene transcription account for the observed differences in

activation responses. A cytokine ELISA failed to detect any IL-4 (data not shown) in 20 microliter supernatant aliquots corresponding to the set analyzed in the IL-2 production assay of these splenocytes shown in Fig. 20A. All splenocyte samples were competent to produce IL-4 in response to stimulation with TPA/ionophore. The absence of IL-4 production by cells from mouse Tg5.3-380 in conjunction with their production of IL-2 suggests that signaling by a tripartite receptor may induce differentiation of Th1 cells. The absence of significant levels of IL-4 production by cells from mice Tg5.3-381 and –382 does not support the possibility that these did not produce high levels of IL-2 due to their differentiating into Th2 cells.

The significant divergence in IL-2 production responses among these three mice stands in contrast to the more stable spectrum displayed by splenocytes from the seven Tg5.3 mice whose individual IL-2 production is shown in high density stimulation (Figure 17). This may be due to differences in the splenocyte handling conditions employed to set up these stimulations. In the stimulation with divergent responses, splenocytes were batch-processed at room temperature which may be optimal for subsequent activation as opposed to processing on ice, however samples further down in the processing queue (Tg5.3-381 and -382), held in air-exposed non-HEPES buffered HBSS longer, were visibly exposed to increasingly alkaline conditions. In the high density stimulation with reproducible responses, splenocytes were serially-processed on ice in HEPES-supplemented HBSS in sealed tubes and were thus maintained at visibly more stable pH. These latter handling conditions may represent a trade-off between optimizing activation potential, as observed in the high proliferation index of splenocytes from mouse Tg5.3-380, versus optimizing stability of handling conditions.

b) Activation of primary splenocytes of Tg5.8 mice by scFv-anti-TNP-CD28-γ

In a preliminary optimization experiment, untreated as well as pre-activated primary splenocytes pooled from Tg5.8 mice produced IL-2 and proliferated in response to immobilized TNP-FγG in a low cell density stimulation. Pre-activation of splenocytes with the anti-CD3 mAb 2C11 prior to stimulation via CR was performed since such pre-activation was shown by Brocker and Karjalainen to be required to enable activation of transgenic T cells via scFvR. Pre-activation of cells with anti-CD3 mAb in low cell density stimulation assays was done as follows. Six-well plates were coated with 5 ug/ml of mAb 2C11 (104) as described above for FγG. Aliquots of 25 $x10^6$ splenocytes in a volume of 4 ml were then stimulated on 2C11 for 48-72h, removed from 2C11 and stimulated in IL-2 (100 U/ml) for an additional 24h. Cells were then washed and aliquots of 10^5 cells were distributed into microtiter plates in a volume of 200 µl/well.

Pre-activated splenocytes were observed to proliferate on Day 2.5 after stimulation with TNP-FγG, when first analyzed, and continued to proliferate until the final time-point on Day 5.5 (Figure 21, top left). Production of IL-2 by pre-activated splenocytes was observed on Day 2.5 and declined to background levels by Day 5.5 (Figure 21, bottom left). Maximal IL-2 production was higher in pre-activated cells than in untreated cells, however their proliferation profiles were similar.

Without pre-activation, induction of proliferation in response to stimulation with plastic-bound TNP-FγG was observed when first analyzed on Day 4 post-stimulation and increased until the final time-point of the assay on Day 6 (Figure 21, top right). Production of IL-2 was evident on Day 1 post-stimulation, reached a peak on Day 2 and declined to background levels by Day 3 (Figure 21, bottom right).

Generation of transgenic mice containing scFV-anti-HER2-CD28-Fcε Receptor γ

To make a triple chimera with HER2 specificity, a transgenic mouse line was generated by pronuclear microinjection of B6 embryos with a construct for expression of (CD3δ)-scFv-anti-HER2-CD28-γ. This line was found to bear 100-150 genomically integrated copies of transgene (Fig. 21A) and splenocytes from these mice were found to express scFv-antiHER2-CD28-γ mRNA as determined by RT-PCR (Figure 22B). Inbred hybrid transgenic mouse lines TgA4-B and –S, the F1 progeny of crosses between TgA4 males with Balb/c or SJL females, respectively, were generated. These mice will be candidates to produce lymphocytes adoptive transfer.

HUMAN LYMPOCYTES REDIRECTED WITH HEREGULIN RECEPTOR BINDING MOLECULES

Development of NDF as a lymphocyte re-directing molecule

To determine whether neuregulin is an effective ligand for preparing targeted lymphocytes, a fused gene encoding the EGF domain of NDF/neuregulin β_and the signaling gamma chain of the FœRI receptor of T cells was constructed and expressed on the surface of the cytotoxic MD.45 27J T cell hybridoma. We utilized the EGF-like domain of NDF/neuregulin as a recognition ectodomain since it is considered to be the binding site of the receptor which is sufficient to stimulate ErbB-2 phosphorylation. An immunoglobulin hinge region, 16 amino acids long and containing three cysteine residues, was inserted to offer additional flexibility for ligand-receptor binding and possibly offer critical disulfide bonds to maintain dimeric structure.

Expression of NDF/neuregulin-γ_chimeric receptor in T cells

Following transfection into the MD.45 27J T cell hybridoma, the neuregulin-hinge-γ expression was tested by Western analysis. The fusion protein was expressed as a dimer, as expected from the disulfide linked cysteine of the γ chain and hinge, running at 16 kDa when immunoblotted under reducing, and at 32 kDa under nonreducing conditions (Fig. 23 A,B). In the absence of antibodies against the NDF moiety, we examined surface expression by surface biotinylating the cell transfectants followed by immunoprecipitation with anti-gamma antibodies. These experiments revealed that the chimeric protein is extracellularly expressed at the expected size of 16 kDa under reducing conditions (Fig. 23 C).

Surface expression of the ErbB receptors on tumor targets

Upon introduction of the chimeric neuregulin- based recognition element, we expected that these transfected T cell hybridoma cells should be specifically triggered, without MHC restriction, to produce IL-2 upon incubation with specific target cells which overexpress the neuregulin binding molecules, HER3 or HER4. We tested several human tumor cell lines for expression of HER1, HER2, HER3, and HER4 receptors in order to establish the specificity of the chimeric neuregulin-gamma chain transfected T cells. MCF-7 and

SKBR3 (breast carcinoma), IGROV and SKOV3 (ovarian carcinoma) and control NIH-3T3 cells were screened by FACS analysis for the presence of these receptors. Results, depicted in Fig. 24, revealed that each tumor target had a unique ErbB receptor family expression profile.

Functional activity of neuregulin-γ chimeric receptor expressing T cells

All of the chimeric neuregulin-γ chain MD.45 27J transfectants were tested for their ability to undergo stimulation by the tumor target cells. The results, depicted in Fig. 25, demonstrate that the chimeric receptor-bearing cells (represented by clones RSIG-12-4, RSIG-12-12, and RSIG-32) are specifically triggered to produce IL-2 when stimulated by target cells which express high levels of ErbB-2 and also express HER3/HER4 (SKBR3 and IGROV), but not by cells overexpressing ErbB-2 alone (SKOV3). All transfectants could undergo stimulation with PMA and ionomycin and none were stimulated by NIH-3T3 fibroblasts, which did not express detectable levels of any of the HER family of receptors. Table 1 summarizes the expression patterns of the tumor cell lines and their stimulation of the neuregulin-γ transfected hybridomas. .

Use of anti-ErbB antibodies to determine neuregulin-γ T cell specificity

To test the specificity of our neuregulin-γ chimeric receptor, we blocked stimulation with antibodies specific to different members of the HER family (Fig. 26). Stimulation of the transfected T cells by SKBR3 cells (expressing HER2 and HER3) was completely inhibited with monoclonal anti-HER3 antibody C105, which blocks the ligand binding site of the HER3 receptor (126). This ErbB-3 directed inhibition was specific for the neuregulin-γ transfected lymphocytes and, as anticipated, had little or no effect on the anti-HER2 N29-γ transfectants. The complete inhibition of neuregulin-γ transfected T cell stimulation by SKBR3 in the presence of anti-HER3 antibody not only demonstrated that the neuregulin directed T lymphocytes are specifically stimulated in the presence of HER3, but it also verified that HER2 alone does not stimulate them. This conclusion was in essence a confirmation of the earlier finding that tumor cells which expresses only HER2 (such as SKOV3), does not activate the transfectant for IL-2 secretion (Fig. 25). SKOV3 cells serve as a good stimulators for T cells expressing the anti-HER2 N29-γ chimeric receptor derived from scFv of anti-HER2 mAb (data not shown).

Nevertheless, the results do not explain why both IGROV and SKBR3, which express direct neuregulin receptors HER3 and/or HER4, could stimulate our neuregulin-γ transfectants whereas MCF-7, shown to express HER3 receptors at levels comparable to SKBR3, could not. The most conspicuous difference between MCF-7 and SKBR3 cell lines is the level of HER2 expression; it is extremely low on MCF-7, and high on SKBR3 (Fig. 24). To check the effect of HER2 expression on neuregulin-γ receptor mediated T cell triggering we used a large panel of anti-HER2 antibodies at our disposal (127) to determine if any had the ability to downregulate the HER2 receptor. Indeed, two antibodies, L288 (data not shown) and L26 reduced the extracellular expression of HER2, while five other antibodies, represented by MAb L140, did not (Fig. 27A). None of the anti-HER2 antibodies cross-react with the HER1, HER3, or HER4 receptors and none compete for the same HER2 epitope ((38) and data not shown). The downregulation of HER2 receptors could be viewed kinetically (Fig. 27B) with the maximum internalization at 2 hours.

We used this panel of anti-HER2 antibodies to test their effects on the SKBR3 stimulation of the neuregulin-directed T lymphocytes. Indeed, antibody L26, representative of the internalizing antibodies, but not antibody L140, representative of the non-internalizing antibodies, could completely inhibit the stimulation (Fig. 28). This L26-mediated inhibition of SKBR3 stimulation was as effective as the inhibition mediated by antibody C105, which blocks the ligand binding site of HER3 (data not shown). Taken together, these results demonstrate that coexpression of both HER2 and HER3 is critical for stimulation of the neuregulin-directed T lymphocytes.

Preparation of retrovirus to transduce tripartite receptors into human lymphocytes

We have developed a retroviral system for the introduction of the chimeric T cell receptors into human lymphocytes. It is a Moloney leukemia virus based system (50) with the chimeric receptor expressed from the retroviral LTR and followed by an IRES enabling the expression of a downstream GFP gene (105, 106). GFP is an efficient method of marking human lymphocytes (107). This transfer vector is packaged in a Ping-Pong system (108) and infected into PG13, a packaging cell line expressing the gibbon ape leukemia virus envelope protein (13, 16, 17, 109). Infection of anti-CD3 and anti-CD28 activated lymphocytes was performed as described previously (10, 110). The packaging cells can be sorted on the basis of the green fluorescence to give a population of cells producing virus. They can also be cloned by sorting cells into individual wells to give a virus producing line (Figure 29).

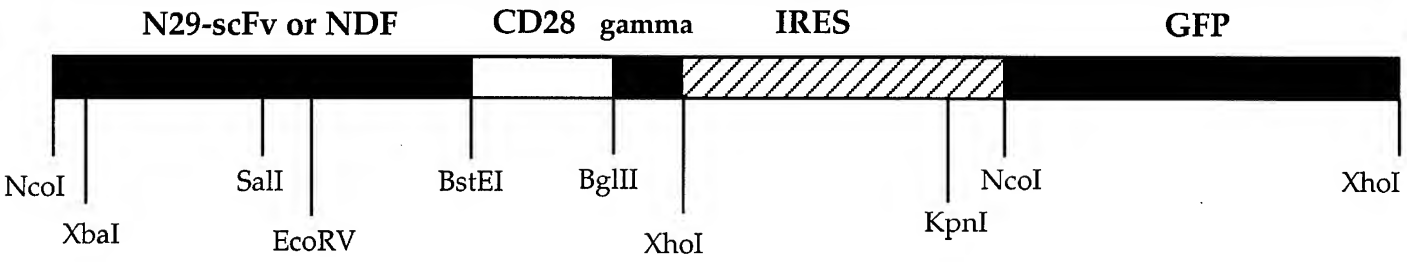
For optimal transduction of human peripheral blood lymphocytes with retroviruses the lymphocytes are activated. They are grown in RPMI with fetal calf serum (FCS) without IL-2 with plastic bound anti-CD3 and anti-CD28 antibodies for 48 hours. They are then infected with supernatant containing virus from the packaging cell line on plates coated with recombinant fibronectin fragments (Retrofectin™). The infection is done in the presence of 50 U/ml of IL-2 for 5 hours. The cells are then grown for 24 hours in RPMI-FCS with 100 U/ml of IL-2 and the infection repeated for another 5 hours. Low concentrations of IL-2 were used to prevent to propagation of natural killer cells. Higher concentrations are used for the production of lymphokine-activated killer (LAK) cells (111).

Two targeting molecules were used in this transfer vector. These targeting molecules were directed towards the heregulin receptor, which is composed of HER2 and HER3 or HER4. One is a scFv targeting HER2 based on the N29 monoclonal antibody (112, 113). Heregulin itself has been used as the targeting molecule for toxins, viruses and cells (5, 114-119). In our case we have a YOL epitope tag to facilitate the detection of chimeric receptor expression by a monoclonal antibody (120).Indeed, human PBL, activated with anti-CD28+CD3 antibodies and transduced by supernatants of retrovector producing packaging cells, expressed the chimeric receptors and GFP (30-40% of the cells were stained, data not shown). Chinese hamster ovary (CHO) cells and 32D, a mouse hematopoietic cell line (121-123), display lower background killing. These cells have been transfected with HER2 together with HER3 (124, 125). When these cells are used as targets, HER2/3 dependent killing is much clearer (Figures 30, 31). Sensitivity is correlated to the level of expression of HER2/3. The CHO cells with HER2 and HER3 contain 60,000 NDF receptors with an affinity of 2 nM(124). The 32D cells with HER2 and HER3 express about 12,000 NDF receptors (125).

We tried different methods for activating the lymphocytes to prime them for killing. Figure 32 shows the results of killing with lymphocytes which have been activated by OKT3, an antibody to CD3, and anti-CD28, or with that antibody combination with added IL-2 or withIL-2 alone. It is apparent that IL-2 stimulation suppressed the non-specific killing leading to specific killing of 32D cells expressing HER2 and HER3 by anti-HER2 directed lymphocytes.

Taken together we have in various stages of development with the lead target, HER2, targeted by human lymphocytes genetically engineered by retroviral transduction of a chimeric scFv-T cell receptor.

Figure 1. ErbB-SPECIFIC CHIMERIC RECEPTOR GENES



N29– Single chain antibody to ErbB2

- Antigen is overexpressed on tumor cells
- Anti-idiotypic antibody is available

NDF- The neuregulin ligand for the ErbB3&4

- The EGF domain is sufficient to bind to surface receptors
- Specific to ErbB3 & 4 homodimers or to heterodimers of ErbB2 and ErbB3

CD28- Co-stimulatory molecule

- Provides co-stimulation
- Prevention of apoptosis
- Heterodimerization
- Spacing scFv from membrane

IRES- Internal Ribosomal Entry Site

- Only one promoter; no promoter interference
- Linked expression

GFP-Green Fluorescent Protein

- Visualization in real time

Figure 2 Junctions

```
        1                                   1
        0                                   1
        3                                   1
                  A           S   L                   A
        W   G     Q     G     T   L   V   T   V   S   S   A
                                T   T
j4      TGG GGT CAA GGA ACC TCA GTC ACC GTC TCC TCA G
j3      TGG GGC CAA GGG ACT CTG GTC ACT GTC TCT GCA G
j2      TGG GGC CAA GGC ACC ACT CTC ACA GTC TCC TCA G
j1      TGG GGC GCA GGG ACC ACG GTC ACC GTC TCC TCA G
JCTN    TGG GGC CAA GGA ACT ACG GTC ACC GTG AAA GGG AAA CAC CTT TGT CCA AGT
         W   G   Q   G   T   T   V   T   V   K   G   K   H   L   C   P   S
CD28     K   S   N   G   T   I   I   H   V   K   G   K   H   L   C   P   S
```

end of CD28 -FcεR gamma chain junction

```
JCTN CCA CCA CGC GAC TTC GCA GCC TAT AGA TCT CAA GTG CGA AAG GCA GCT ATA
      P   P   R   D   F   A   A   Y   R   S   Q   V   R   K   A   A   I
CD28  P   P   R   D   F   A   A   Y   R   S   *
FCEG                      C   R   L   K   I   Q   V   R   K   A   A   I
```

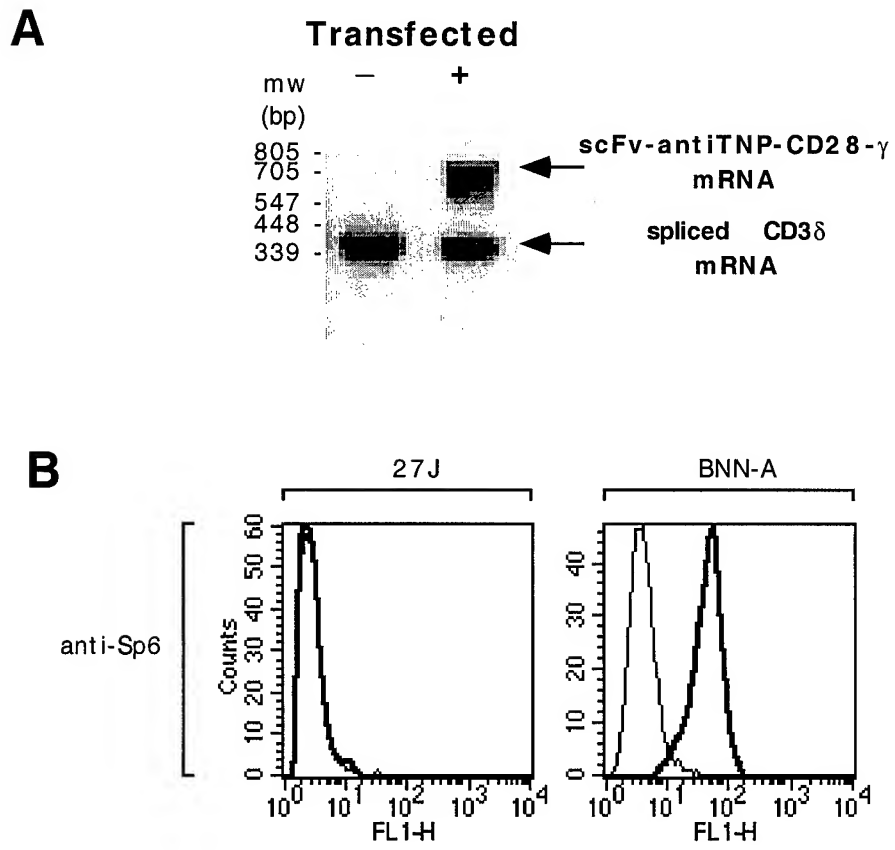


Figure 3. Hybridoma transfectant expresses scFv-antiTNP-CD28-γ mRNA and scFv-antiTNP-CD28-γ surface proteins. (A) Complementary DNA was PCR amplified with scFv-antiTNP-CD28-γ specific primers. To control for amplification from spliced RNA and to normalize samples, cDNA was co-amplified with intron-flanking CD3δ primers which generate a 345 bp or a 759 bp amplification product from spliced or unspliced CD3δ mRNA, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining. (B) Immunofluorescence analysis of scFv-antiTNP-CD28-γ surface expression. Primary staining reagents, biotinylated anti-(anti-TNP mAb) Sp6-idiotype mAb GK-20.5 (bold line) or irrelevant staining reagent, biotinylated anti-DNP mAb U-7.6; secondary detection reagent, FITC-streptavidin.

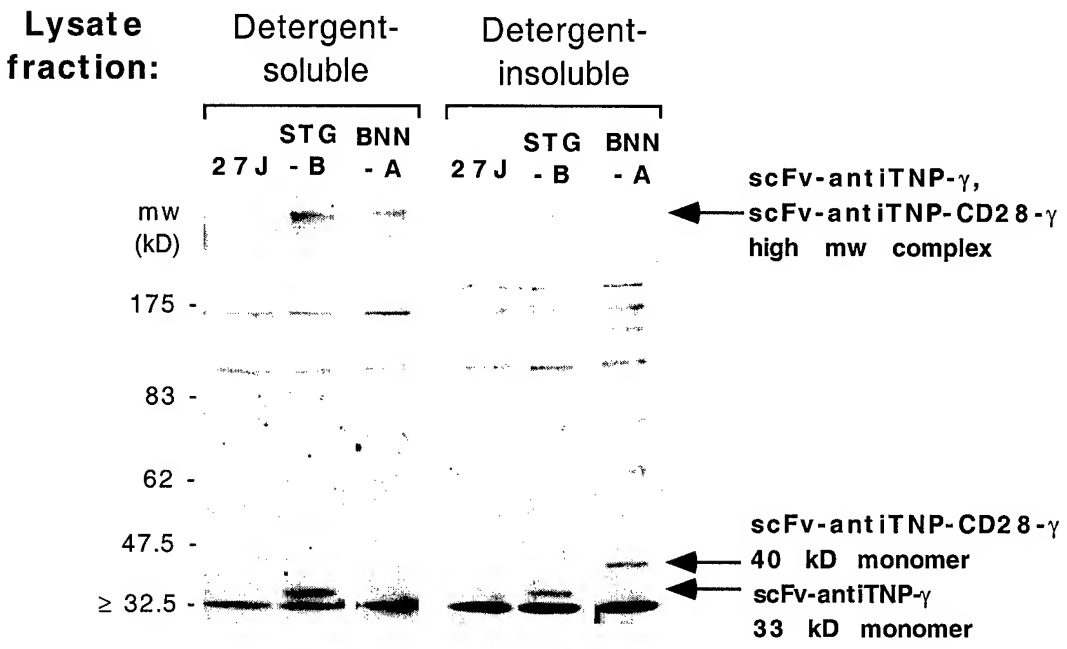


Figure 4. Subcellular localization of scFv-antiTNP-CD28-γ protein by Western immunoblotting. Detergent soluble protein fraction represents NP-40 lysate supernatant and detergent insoluble fraction was obtained homogenization of NP-40 insoluble precipitate in RIPA lysis buffer. Lysate proteins were separated by SDS-PAGE under reducing conditions, blotted onto nitrocellulose and stained with rabbit anti-γ serum followed by staining with HRP-protein A and visualized by ECL. The hybridoma transfectant STG-B expressing scFv-antiTNP-γ and the parental untransfected line 27J were used as positive and negative controls, respectively.

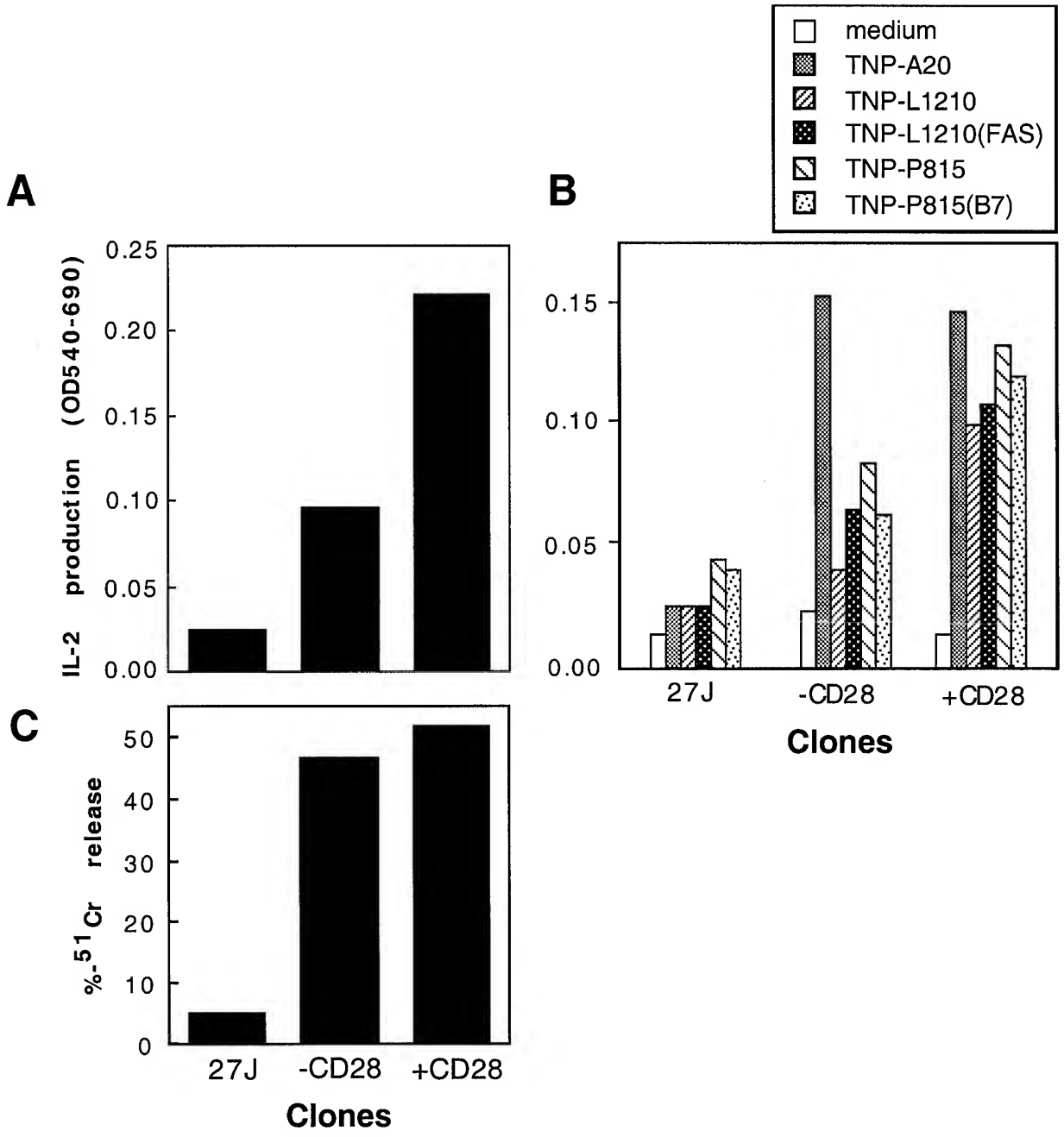


Figure 5. Hybridoma transfectant expressing Sp6-CD28-γ is activated to perform effector functions in response to stimulation with TNP. (A) Stimulation of IL-2 production by plastic-immobilized TNP-FγG. (B) Stimulation of IL-2 production by TNP-modified cells. Target cells; A20, BALB/c B lymphoma-derived cell line; L1210, murine leukemia; L1210(FAS), L1210-derived transfectant expressing fas; P815, murine mastocytoma; P815(B7), P815-derived transfectant expressing B7. (C) Cytolysis of TNP-modified A20 cells. 27J, untransfected parental hybridoma effector negative control; -CD28 is a hybridoma transfectant expressing scFv-anti-TNP-γ.

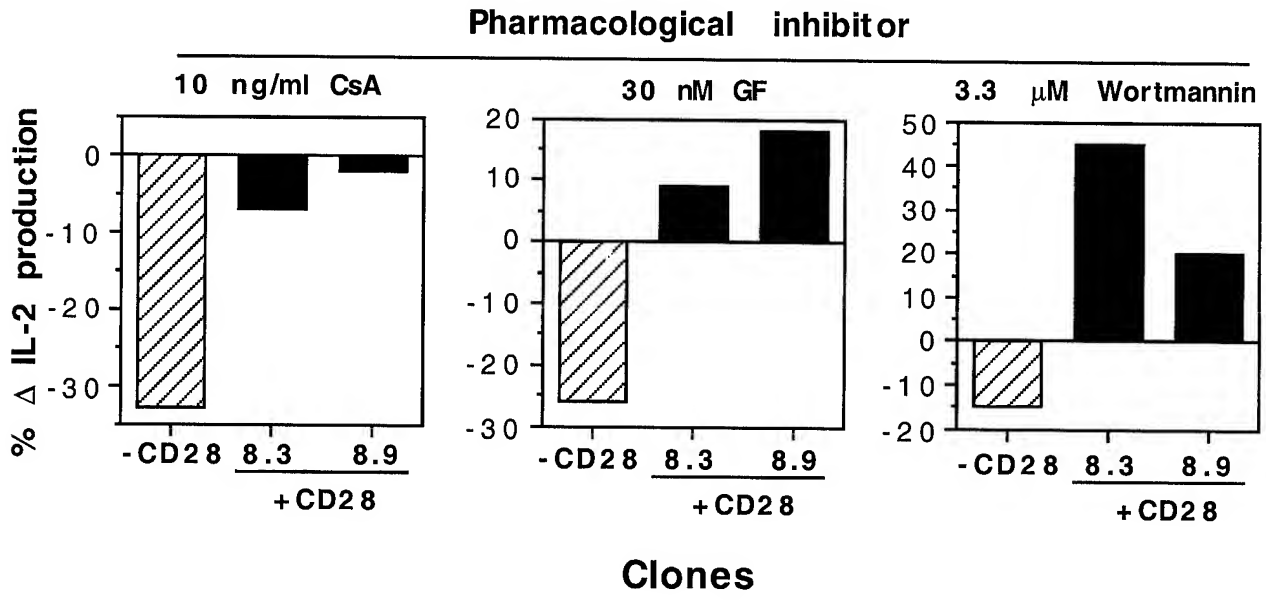


Figure 6. Signaling for IL-2 production by scFv-antiTNP-CD28-γ contains a CD28-specific signaling component. Hybridoma transfectant clones expressing scFv-antiTNP-γ (STG-B) or scFv-antiTNP-CD28-γ (BNN-A subclones 8.3 and 8.9) were stimulated to produce IL-2 by immobilized TNP-FgG in the presence or absence of pharmacological inhibitors. The untransfected parental clone 27J was used as negative effector control and medium and TPA+ionophore were used as negative and positive stimulator controls, respectively. Stimulations were performed with 10^6 cells/well in 24-well plates in a volume of 1 ml. Cells were stimulated for 24h and triplicate supernatant aliquots were harvested. The IL-2 content was determined via CTLL IL-2 bioassay and quantitation of CTLL proliferation by MTT. To compensate for the effect of residual inhibitor on the growth response of CTLL to IL-2, the effect of 27J + inhibitor-conditioned supernatants on the growth response of CTLL to a mid-range concentration of IL-2 was determined. Only GF had such an effect and a multiplication factor (MF) of 1.2 (vs 1.0 for CsA and wortmannin) was used to normalize quantitation of IL-2 production by this inhibitor. The BNN-A clones whose results are shown are those which produced a quantifiable mid-range quantity of IL-2 upon stimulation in the absence of inhibitor, similar to that produced by the reference scFv-antiTNP-γ transfectant clone STG-B.
(%Δ IL-2 production) = 100 x (IL-2 production + inhibitor)x(MF)/(IL-2 production - inhibitor).

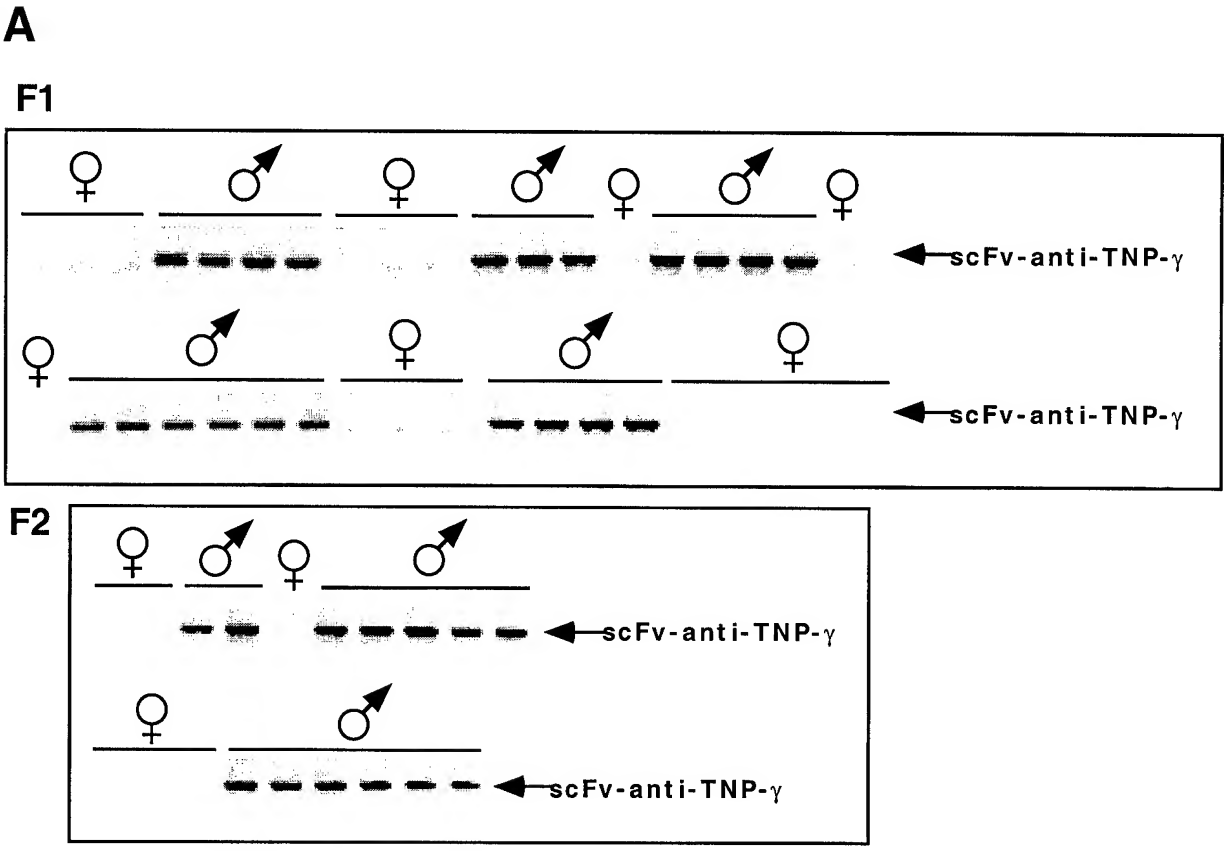
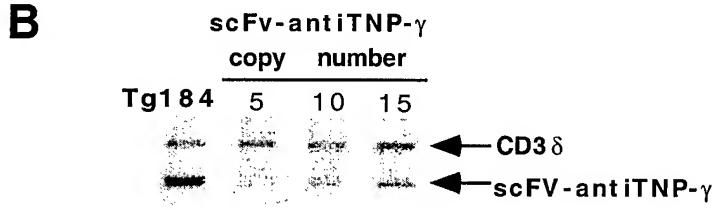


Figure 7. Mouse line Tg184 transgenic for (CD3δ)-scFv-antiTNP-γ bears ~50 copies of transgene integrated in the Y chromosome. (A) Male-specific PCR-amplification of scFv-antiTNP-γ sequences from genomic DNA of F1 and F2 progeny descended from the founder male of line Tg184.



B) Estimation of the number of genomically integrated copies of transgene. Copy number estimation was performed via semi-quantitative PCR amplification of a scFv-antiTNP-γ specific sequence from genomic DNA and from copy number standards composed of C57BL/6 DNA containing dosed quantities of transgene-encoding vector. Amplification products were separated by agarose gel electrophoresis and visualized by EtBr staining.

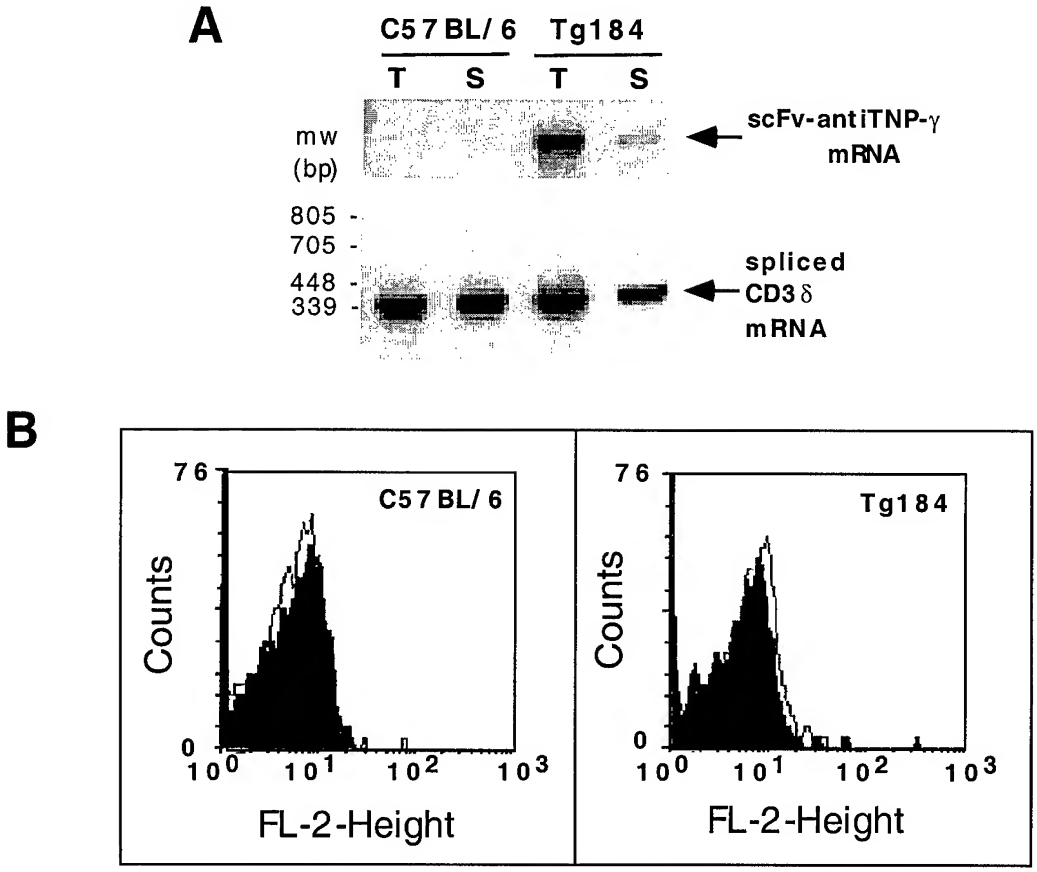


Figure 8. Expression of (CD3δ)-scFv-anti-TNP-γ transgene in splenic T cells of Tg184 mice. (A) Splenic T cells from transgenic mouse line Tg184 express scFV-antiTNP-γ mRNA. Complementary DNA from splenic T cells (S) purified by panning on immobilized Ig and thymocytes (T) was PCR-amplified with Sp6-γ specific primers. To control for amplification from mature spliced mRNA and to normalize samples cDNA was amplified with intron-flanking CD3δ primers which generate a 345 bp or a 759 bp amplification product from spliced or unspliced CD3δ mRNA, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining. (B) Immunofluorescence analysis of scFv-antiTNP-γ surface expression. Primary staining reagent, anti-Sp6-idiotype mAb GK-20.5; irrelevant primary staining reagent, anti-DNP mAb U-7.6 (solid fill); secondary detection reagent; PE-donkey anti-mouse IgG polyclonal Ab.

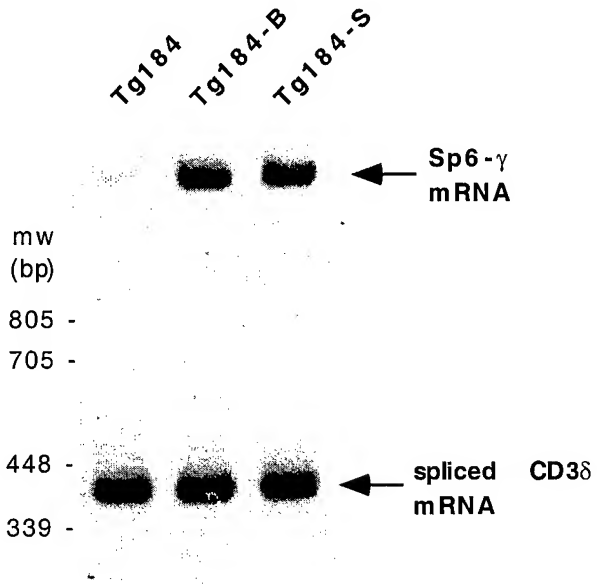


Figure 9. Hybrid Tg184-B and Tg184-S splenocytes express scFv-antiTNP-γ mRNA at higher levels than Tg184. Complementary DNA prepared from whole splenocytes was PCR amplified with scFv-antiTNP-γ specific primers (top). Amplification with intron-flanking CD3δ-specific primers was performed to control for amplification from mature spliced mRNA and to normalize samples (bottom). These primers amplify a 345 bp or a 759 bp sequence from cDNA generated from spliced or unspliced CD3δ sequences, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining.

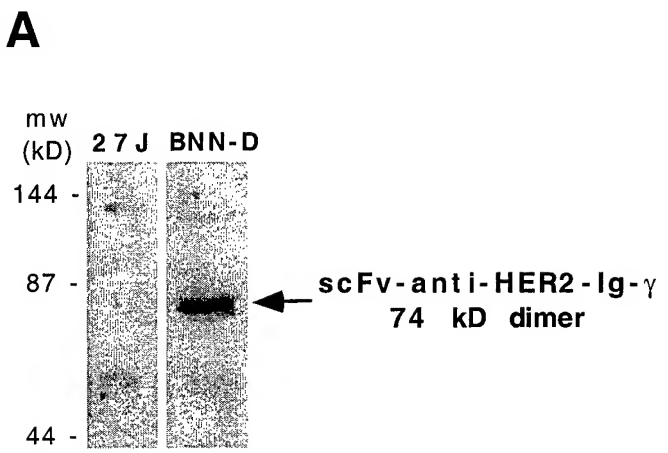
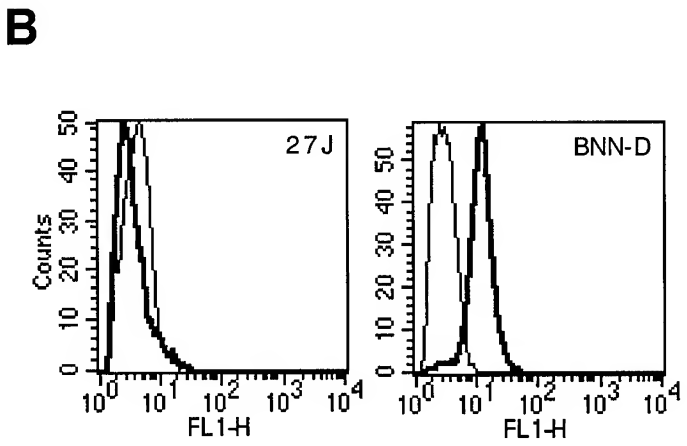


Figure 10. Hybridoma transfectant BNN-D expresses CD3δ-regulated scFV-antiHER2-Ig-γ protein. (A) Immunoblotting analysis of protein expression. Lysate proteins were separated by SDS-PAGE under non-reducing conditions, blotted onto nitrocellulose and stained with rabbit anti-N29 (anti-HER2 mAb) serum followed by staining with HRP-protein A and visualized by ECL. The untransfected parental line 27J was used as negative control.



(B) Immunofluorescence analysis of receptor surface expression. Primary staining reagent, rabbit anti-N29 (anti-HER2 mAb) serum (bold); irrelevant primary staining reagent, normal rabbit serum; secondary detection reagent, FITC-goat anti-rabbit IgG Ab.

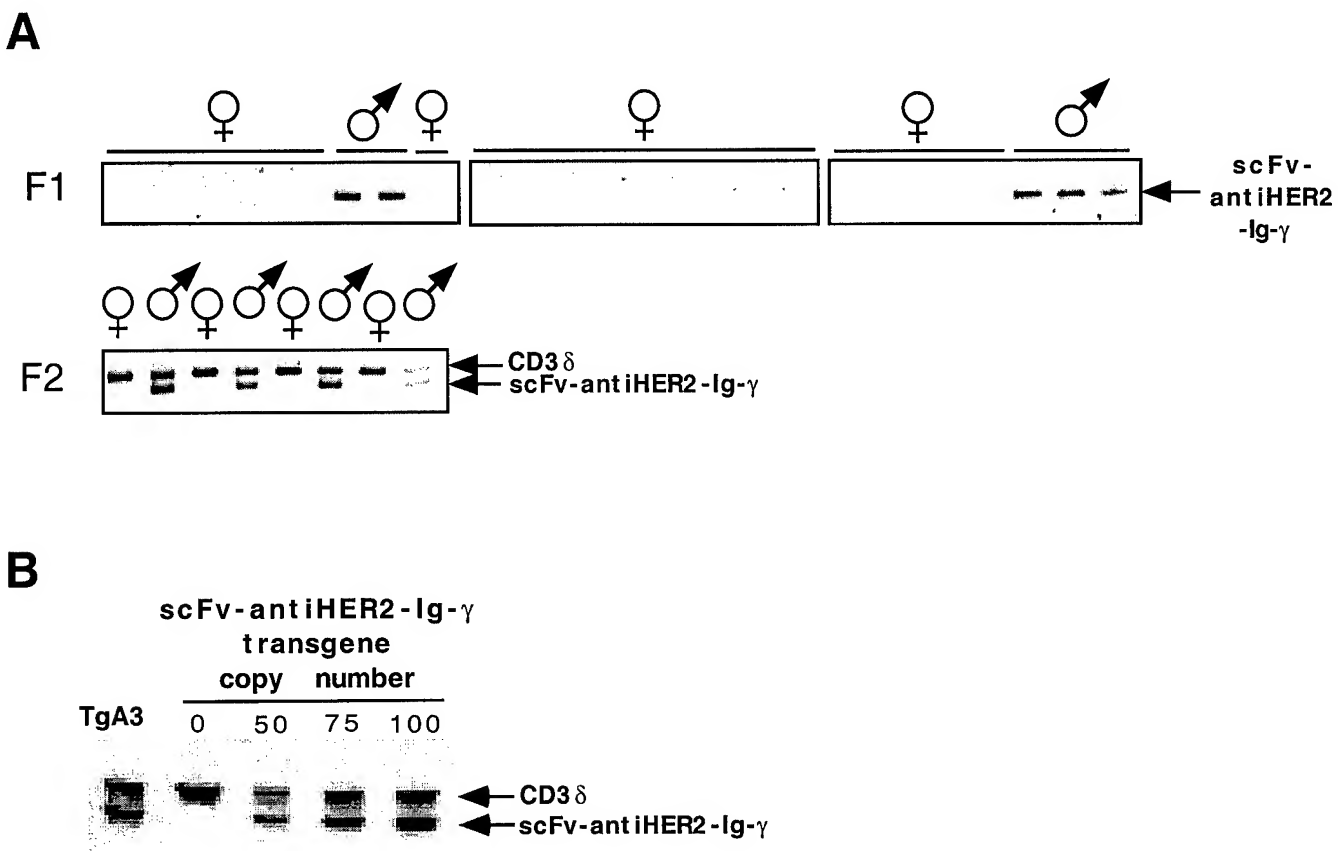


Figure 11. Mouse line TgA3 transgenic for (CD3δ)-scFv-antiHER2-Ig-γ bears 75-100 copies of transgene integrated in the Y chromosome. (A) Male-specific PCR-amplification of scFv-antiHER2-Ig-γ sequences from genomic DNA of F1 and F2 progeny descended from the founder male of line TgA3. (B) Estimation of the number of genomically integrated copies of transgene. Copy number estimation was performed via semi-quantitative PCR amplification of a scFv-antiHER2-Ig-γ specific sequence from genomic DNA and from copy number standards composed of C57BL/6 DNA containing dosed quantities of transgene-encoding vector. Amplification products were separated by agarose gel electrophoresis and visualized by EtBr staining.

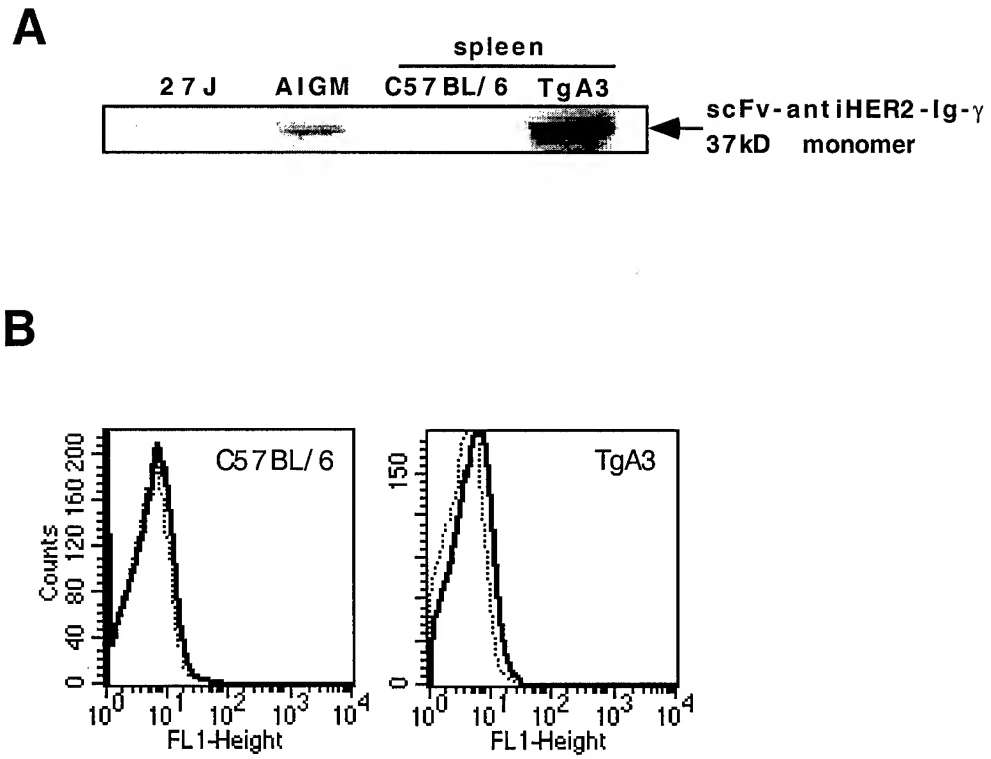


Figure 12. Expression of transgene protein in T cells from mouse line TgA3 transgenic for (CD3δ)-scFv-antiHER2-Ig-γ . (A) Immunoblotting analysis of protein expression in splenocytes. Lysate proteins were separated by SDS-PAGE under reducing conditions, blotted onto nitrocellulose and stained with rabbit anti-N29 (anti-HER2 mAb) serum followed by staining with HRP-protein A and were visualized by ECL. The scFv-antiHER2-Ig-γ expressing hybridoma transfectant AIGM and its untransfected parental line 27J were used for positive control. (B) Immunofluorescence analysis of surface expression in thymocytes of TgA3 mice. Primary staining reagent, rabbit anti-N29 (anti-HER2 mAb) serum; irrelevant primary staining reagent; normal rabbit serum (dotted line); secondary detection reagent, FITC-goat anti-rabbit IgG Ab.

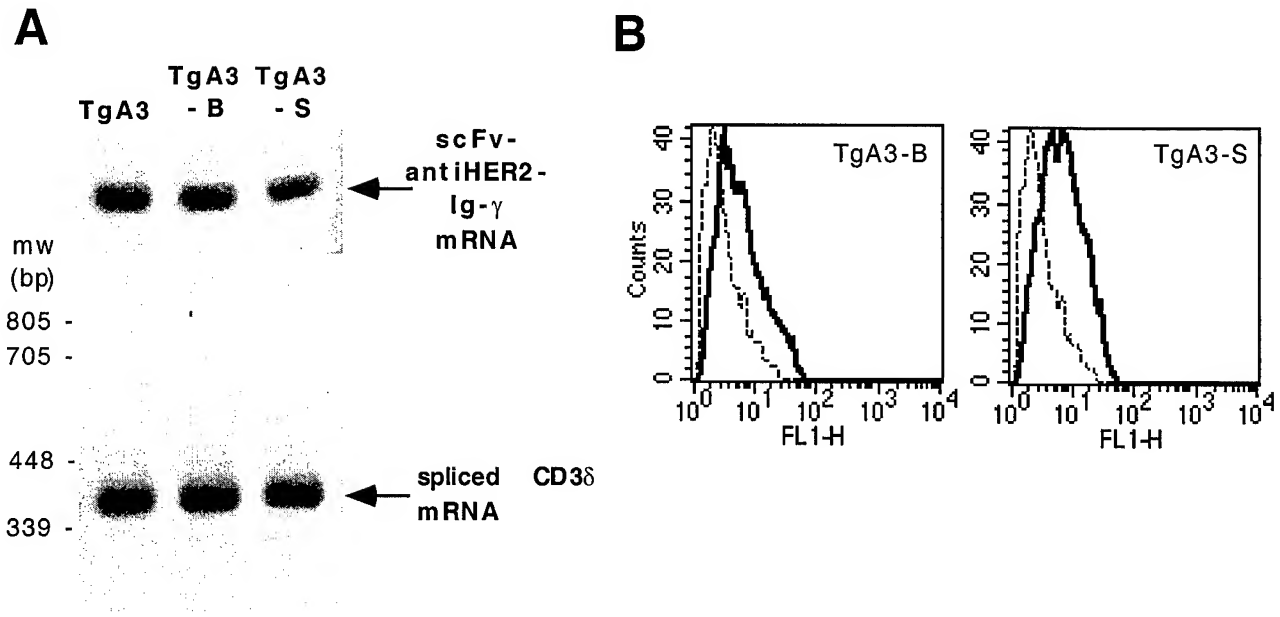


Figure 13. Expression of (CD3δ)-scFv-antiHER2-Ig-γ in splenocytes from hybrid TgA3-B and TgA3-S mice. (A) Splenocytes from TgA3-B and TgA3-S mice express scFv-antiHER2-Ig-γ mRNA. Complementary DNA was PCR-amplified at sub-saturating levels with scFv-antiHER2-Ig-γ specific primers (top). To control for amplification from mature spliced mRNA and to normalize samples, cDNA was amplified with intron-flanking CD3δ-specific primers (bottom). These primers amplify a 345 bp or a 759 bp sequence from cDNA generated from spliced or unspliced CD3δ mRNA, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining. (B) Splenocytes from hybrid TgA3-B and TgA3-S mice express higher levels of scFv-antiHER2-Ig-γ surface protein than TgA3. For immunofluorescence analysis splenocytes were stained with rabbit anti-N29 (anti-HER2 mAb) serum followed by staining with FITC-goat anti-rabbit IgG Ab. Shown here are histogram overlays of the indicated hybrid cells (solid line) over TgA3 cells (dashed line).

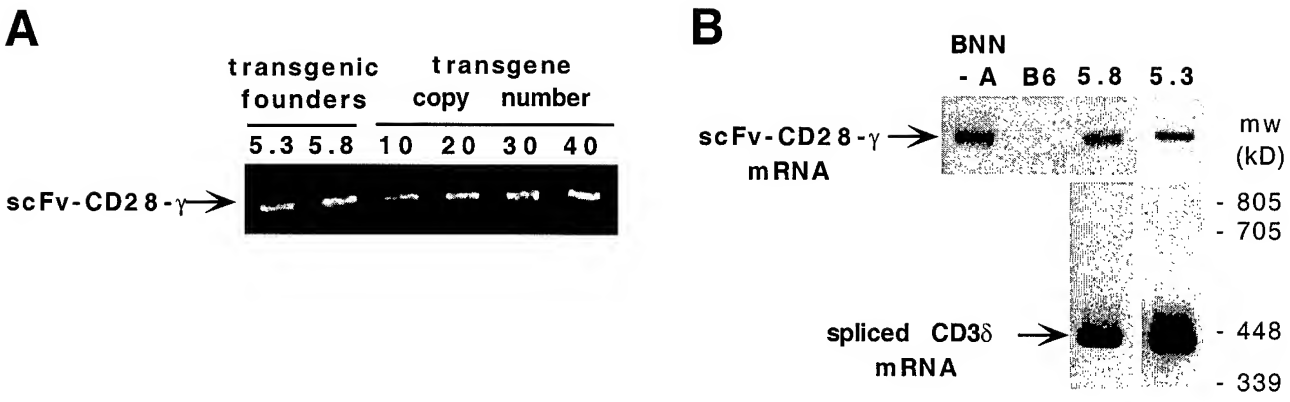


Figure 14. Mouse lines Tg5.3 and Tg5.8 transgenic for (CD3δ)-scFv-antiTNP-CD28-γ have ~40 and ~60 integrated copies of transgene, respectively, and express transgene mRNA. (A) Estimation of genomic transgene copy number. Copy number estimation was performed via semi-quantitative PCR amplification of a scFv-antiTNP-CD28-γ specific sequence from genomic DNA and from copy number standards composed of C57BL/6 genomic DNA containing dosed quantities of transgene-encoding vector. (B) Expression of scFv-antiTNP-CD28-γ mRNA in Tg5.3 and Tg5.8 splenocytes. Complementary DNA was PCR amplified with scFv-antiTNP-CD28-γ specific primers (top). To control for amplification from mature spliced mRNA and to normalize samples, cDNA was amplified with intron-flanking CD3δ primers (bottom). These primers generate a 345 bp or a 759 bp amplification product from mature spliced mRNA or from unspliced sequences, respectively. PCR reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining.

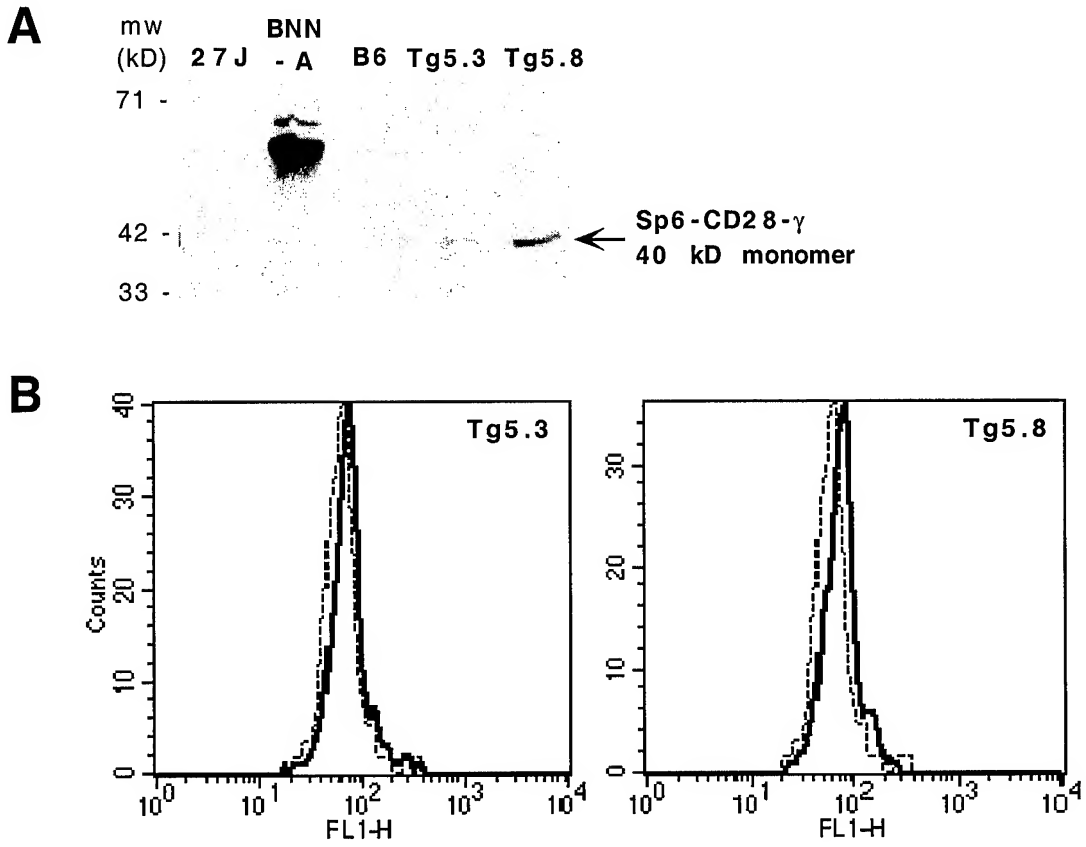


Figure 15. Mouse lines Tg5.3 and Tg5.8 transgenic for (CD3δ)-scFv-antiTNP-CD28-γ express transgene protein. (A) Expression of scFv-antiTNP-CD28-γ protein in Tg5.3 and Tg5.8 splenocytes. Detergent lysates were immunoprecipitated with anti-Sp6 (anti-TNP mAb) idiotype mAb GK20.5 and protein was separated by SDS-PAGE under reducing conditions. Protein was blotted onto nitrocellulose and stained with rabbit anti-γ serum followed by staining with HRP-protein A and visualized by ECL. The scFv-antiTNP-CD28-γ expressing T cell hybridoma transfectant BNN-A and its untransfected parental line 27J were used for positive control. Non-transgenic C57BL/6 (B6) cells were used as negative primary lymphocyte controls. Each lane contains lysate from 50 x 10^6 primary lymphocytes or 6 x 10^6 hybridoma cells. (B) Immunofluorescence analysis of scFv-antiTNP-CD28-γ surface expression in transgenic thymocytes. Cells from the indicated transgenic strain (solid line) and from non-transgenic C57BL/6 mice (dashed line) were stained with biotinylated GK-20.5 and secondarily with FITC-streptavidin.

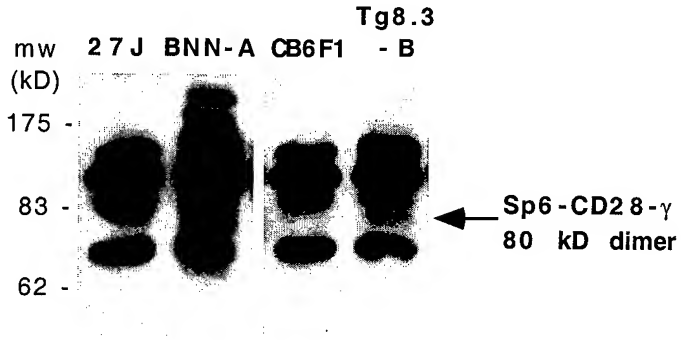


Figure 16. Expression of (CD2)-scFv-antiTNP-CD28-γ protein in T cells of Tg8.3-B mice. Thymocytes of Tg8.3-B mice were analyzed for expression of transgene protein by immunoprecipitation and Western immunoblotting analysis. Detergent lysates were immunoprecipitated with anti-Sp6 (anti-TNP mAb)idiotype mAb GK20.5, separated by SDS-PAGE under partially reducing conditions (required to maintain Sp6 detection epitope) and blotted onto nitrocellulose. Blots were stained with GK20.5 mAb followed by secondary staining with HRP-coupled goat-anti-mouse IgG Ab and specific protein was visualized by ECL. The scFv-antiTNP-CD28-γ expressing T cell hybridoma transfectant BNN-A and its untransfected parental line 27J were used for positive control. Non-transgenic CB6F1 cells were used as negative primary lymphocyte controls. Each lane contains lysate from 50 x 10^6 primary lymphocytes or 5 x 10^6 hybridoma cells.

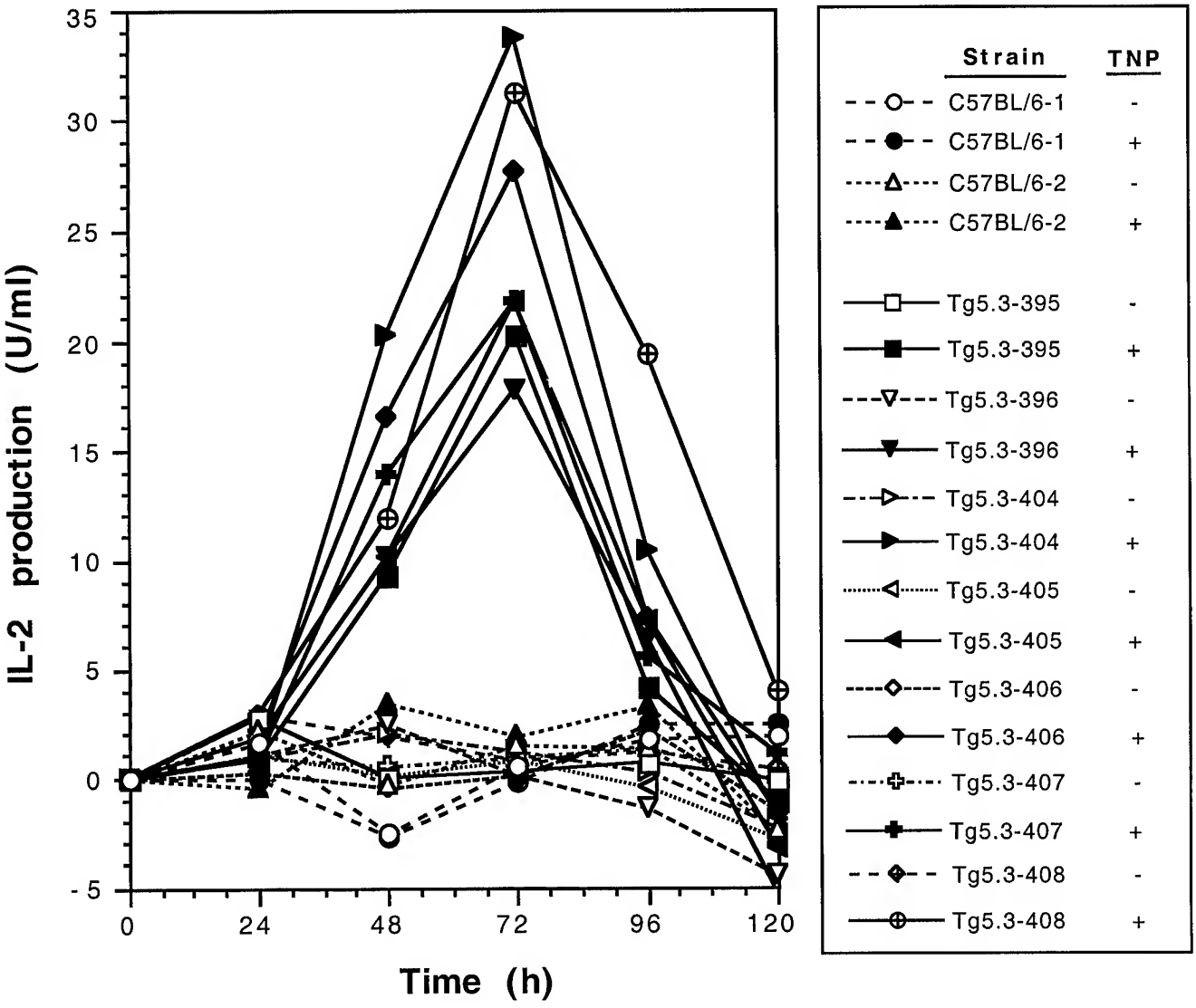


Figure 17. Untreated primary splenocytes from mouse line Tg5.3 transgenic for scFv-antiTNP-CD28-γ produce IL-2 in response to TNP stimulation. Data were derived from parallel stimulations of seven Tg5.3 and two non-transgenic C57BL/6 mice at high cell density.

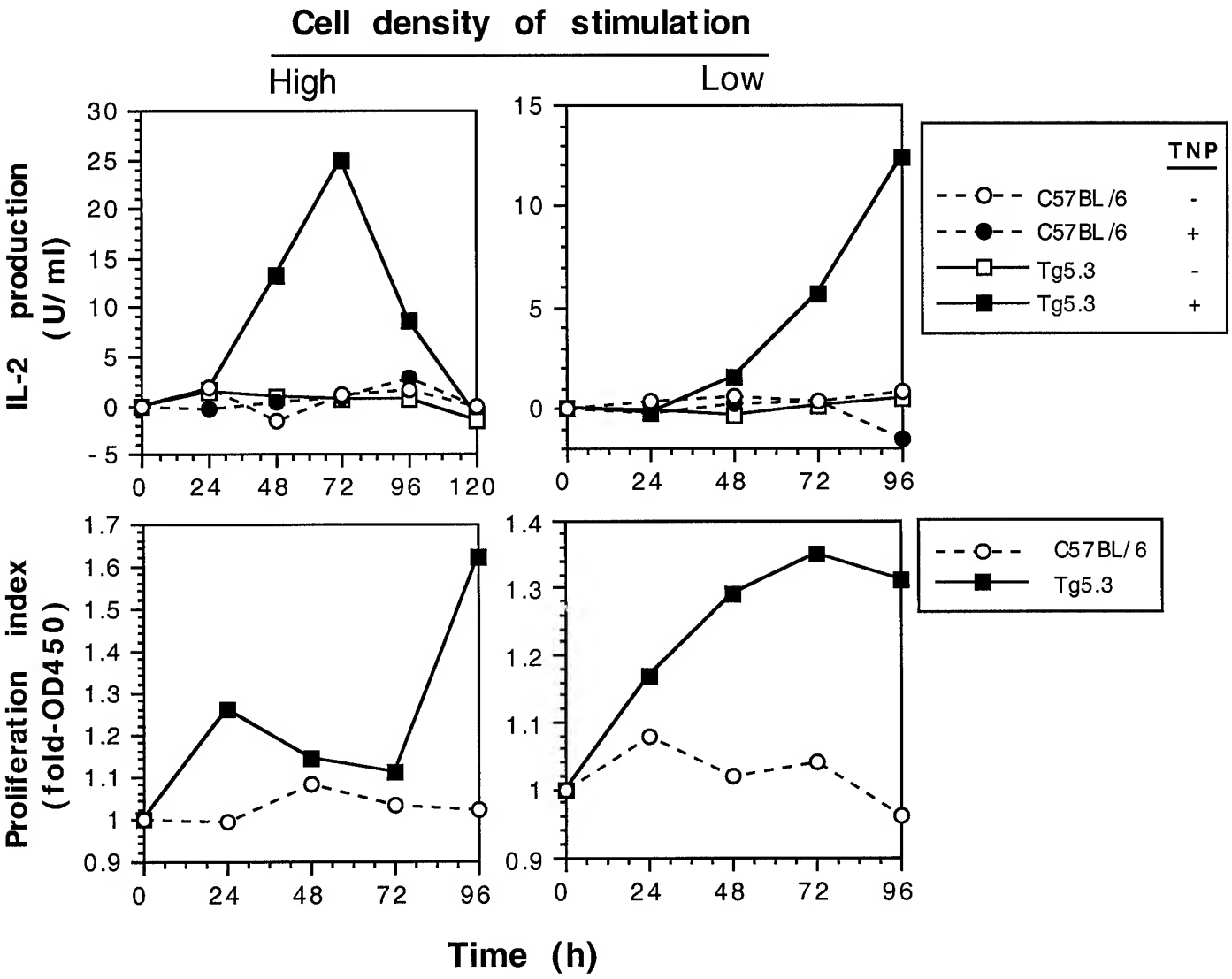


Figure 18. Untreated primary splenocytes from mouse line Tg5.3 transgenic for scFv-antiTNP-CD28-γ proliferate and produce IL-2 in response to TNP stimulation. Data represents average values obtained from parallel stimulations of two non-transgenic C57BL/6 mice and seven Tg5.3 mice.

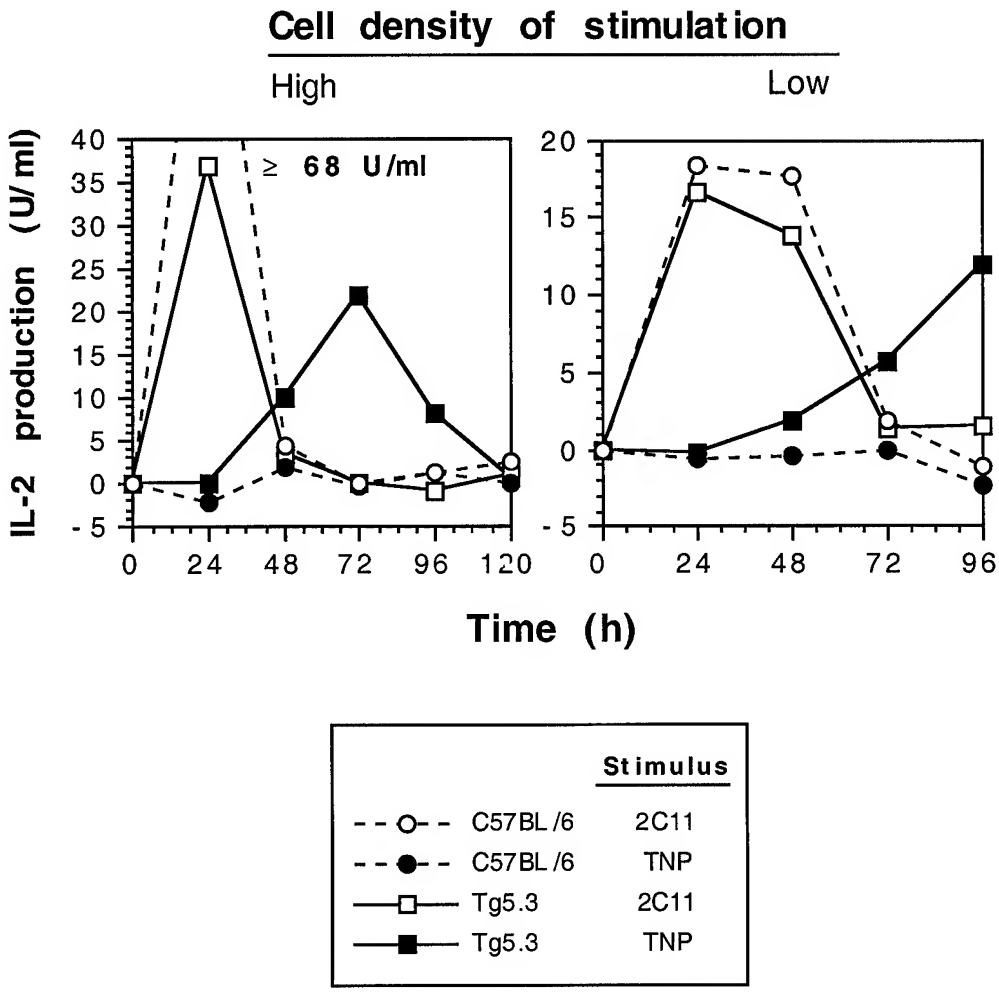


Figure 19. Comparison of IL-2 production by primary Tg5.3 splenocytes transgenic for scFv-antiTNP-CD28-γ in response to stimulation with TNP or 2C11. Data shown represents average values derived from parallel stimulations of two non-transgenic C57BL/6 mice, and seven and five Tg5.3 mice at low and high cell density, respectively.

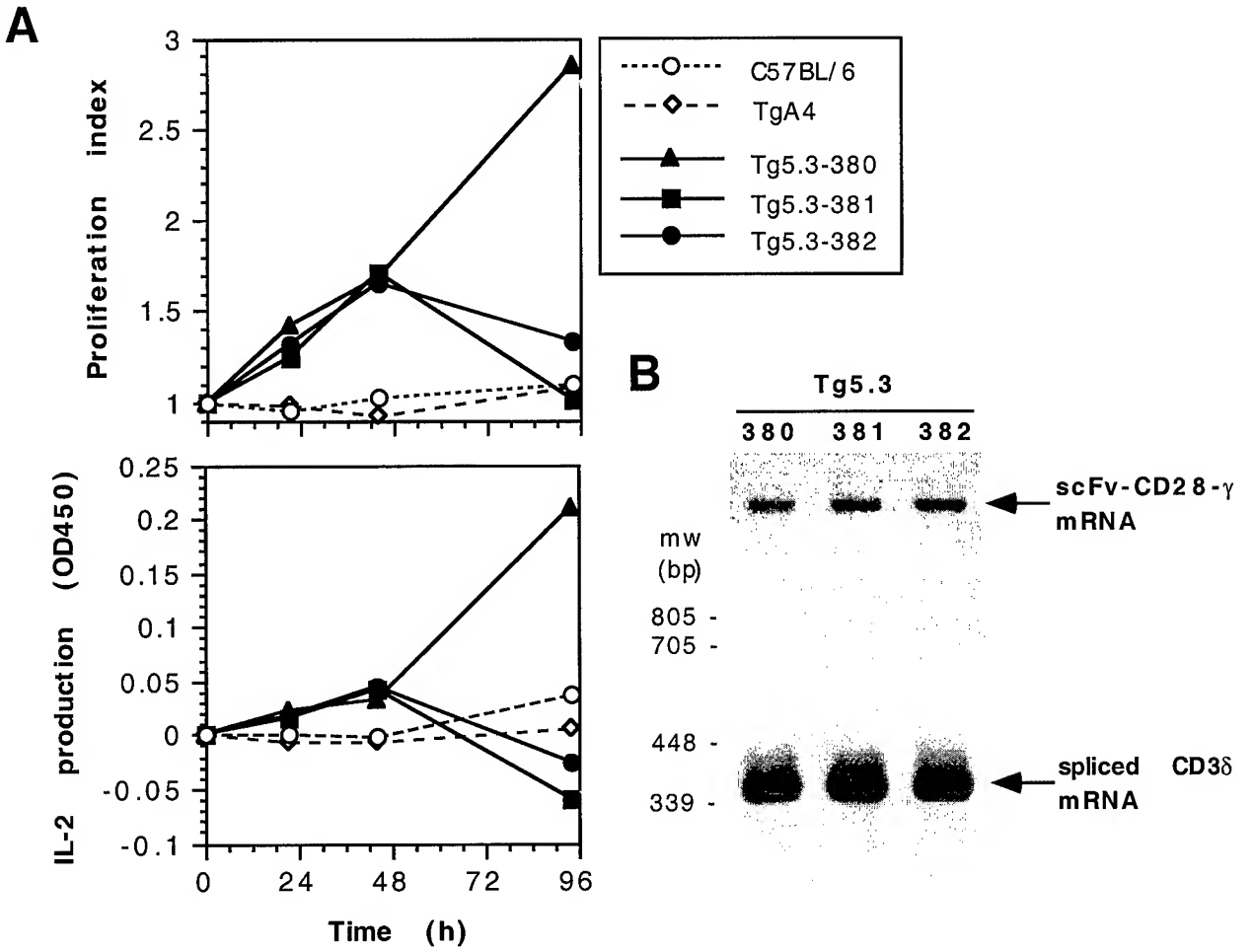


Figure 20. Untreated primary splenocytes from mouse line Tg5.3 transgenic for scFv-antiTNP-CD28-γ are activated in response to TNP. (A) Stimulation assay at low cell density of primary splenocytes from mouse line Tg5.3. Mouse line TgA4 transgenic for scFv-antiHER2-CD28-γ was used as irrelevant Fv control. Data shown was obtained in a preliminary experiment. (B) Splenocytes from mice Tg5.3-380, -381 and -382 transcribe similar levels of scFV-antiTNP-CD28-γ mRNA. Complementary DNA was PCR-amplified to sub-saturating levels with scFv-antiTNP-CD28-γ specific primers (top). Amplification with intron-flanking CD3δ–specific primers was performed to control for amplification from mature spliced RNA and to normalize samples (bottom). These primers amplify a 345 bp or a 759 bp sequence from cDNA generated from spliced or unspliced CD3δ mRNA, respectively. Reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining.

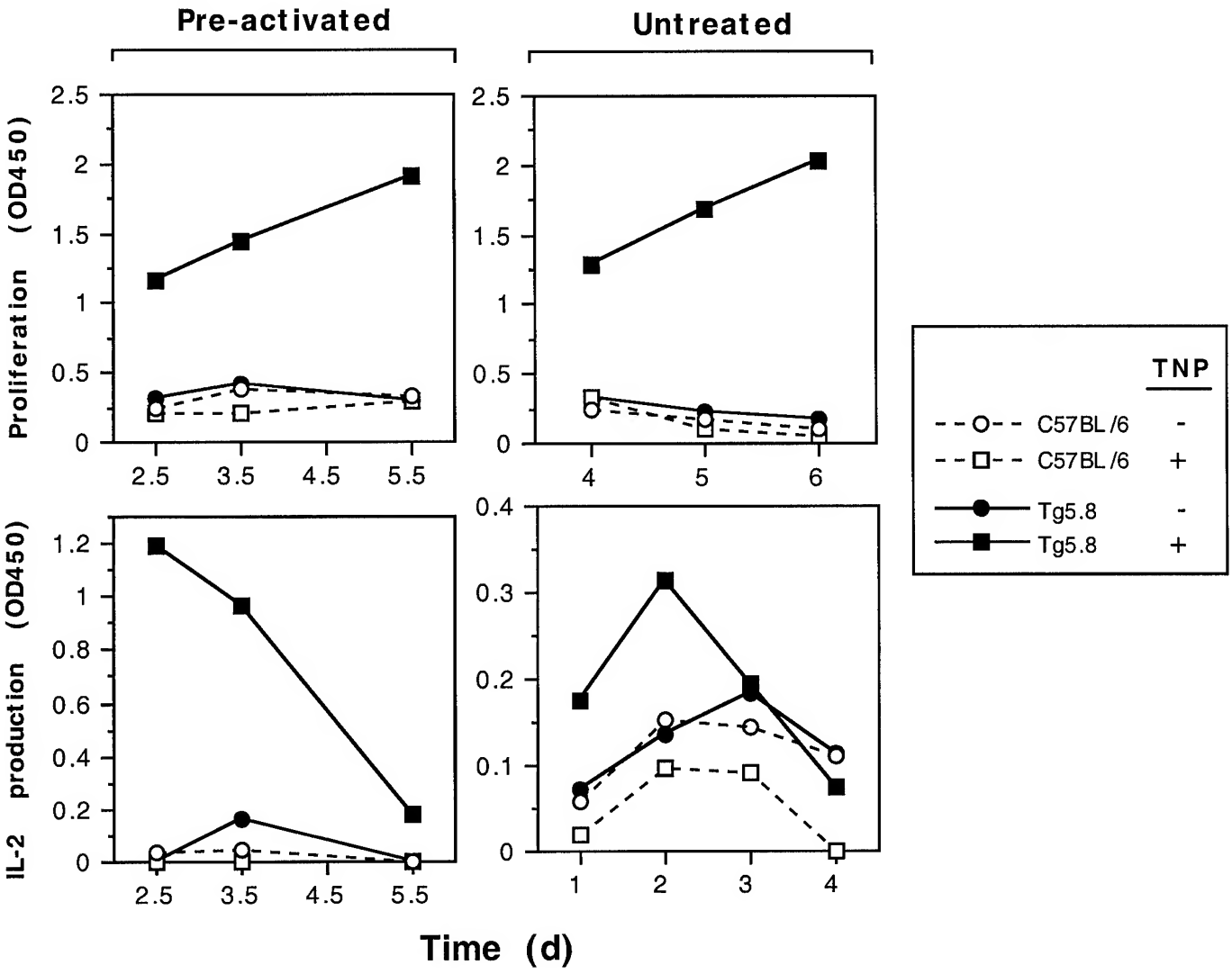


Figure 21. Untreated and pre-activated primary splenocytes from mouse line Tg5.8 transgenic for scFv-antiTNP-CD28-γ are activated in response to TNP. Data was obtained from stimulation at low cell density of splenocytes pooled from two mice. For pre-activation, splenocytes were incubated on immobilized anti-CD3 mAb 2C11 for 48-72 h followed by 24 h of IL-2 stimulation prior to stimulation with TNP. In comparison to the other figures, the proliferation index is the value of the points indicated by the squares divided by the value of the points indicated by the circles.

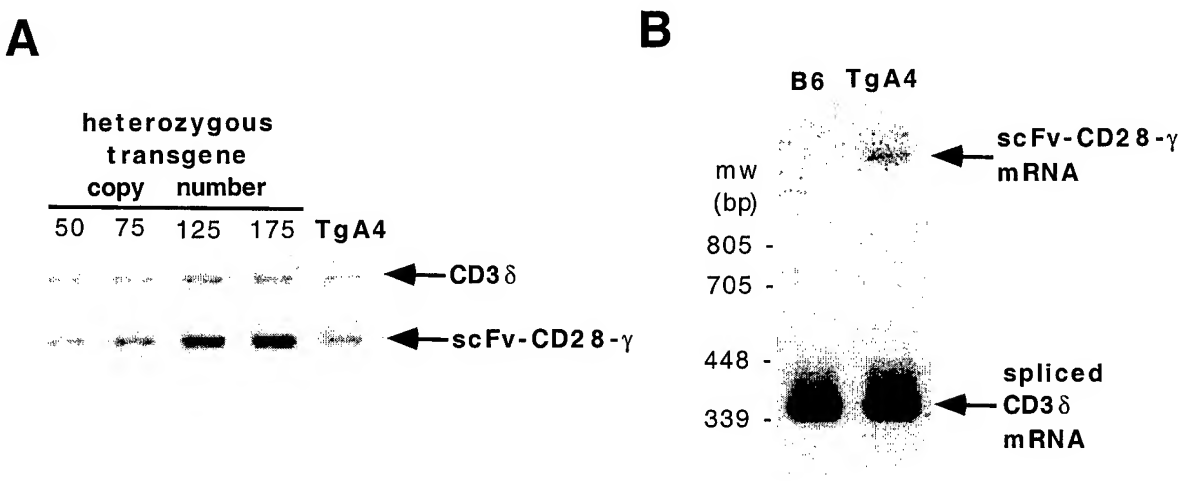


Figure 22. Mouse line TgA4 transgenic for (CD3δ)-scFv-antiHER2-CD28-γ bears 100-150 (homozygous) integrated copies of transgene and expresses transgene mRNA. (A) Estimation of genomic transgene copy number. Copy number estimation was performed via semi-quantitative PCR amplification of a scFv-antiHER2-CD28-γ specific sequence from genomic DNA and from copy number standards composed of C57BL/6 (B6) genomic DNA containing dosed quantities of transgene-encoding vector. Samples were co-amplified with CD3δ-specific primers to normalize template quantitation. (B) Splenocytes from mouse line TgA4 express scFv-antiHER2-CD28-γ mRNA. Complementary DNA was PCR amplified with scFv-antiHER2-CD28-γ specific primers (top). To control for amplification from spliced RNA and to normalize samples cDNA was separately amplified with intron-flanking CD3δ primers (bottom). These primers generate a 345 bp or a 759 bp amplification product from mature spliced mRNA or from unspliced sequences, respectively. PCR reaction products were separated by agarose gel electrophoresis and visualized by EtBr staining.

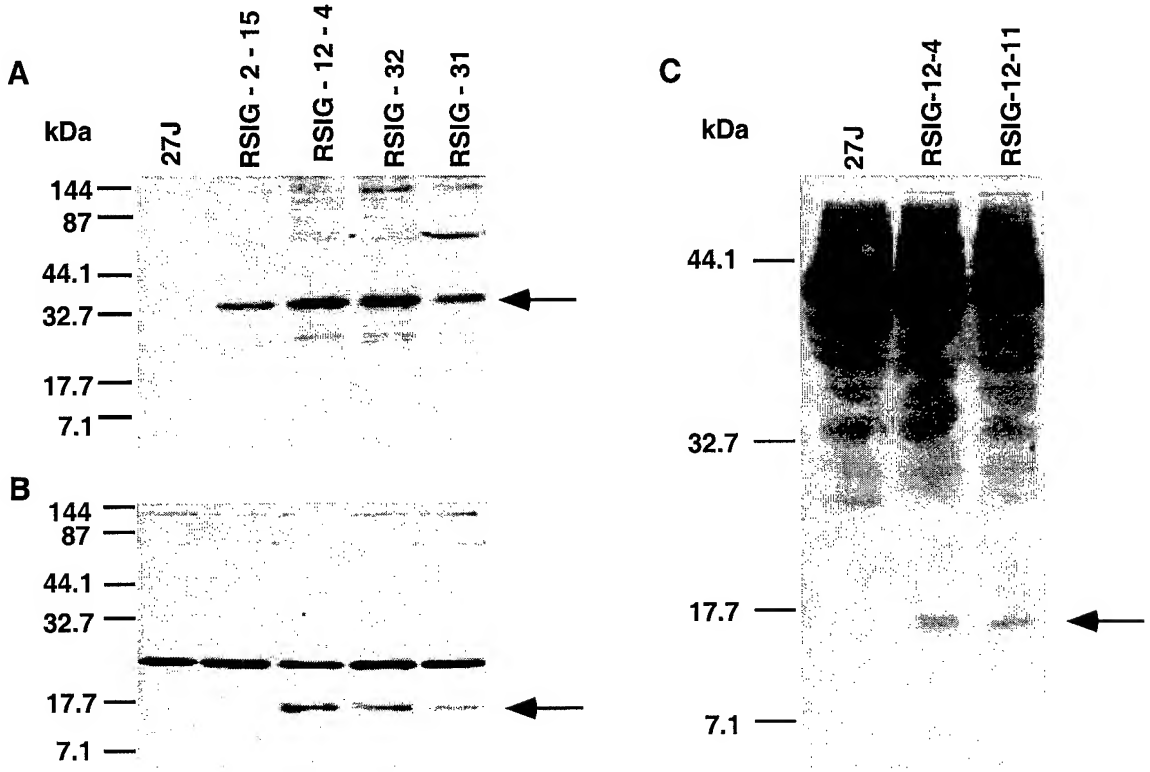


Figure 23. Expression of the neuregulin-γ construct. Immunoblot analysis using anti-gamma antibody of lysates prepared from either the MD.45 27J hybridoma or transfectants. (**A**) Non-reduced and (**B**) reduced 10% acrylamide gels. (**C**) Surface expression of the chimeric neuregulin-based receptor in transfected MD.45 27J cells. Cells (25 x 10^6) were surface biotinylated, immunoprecipitated with anti-Fcε γ chain antibodies, and immunoblotted with peroxidase-labeled streptavidin.

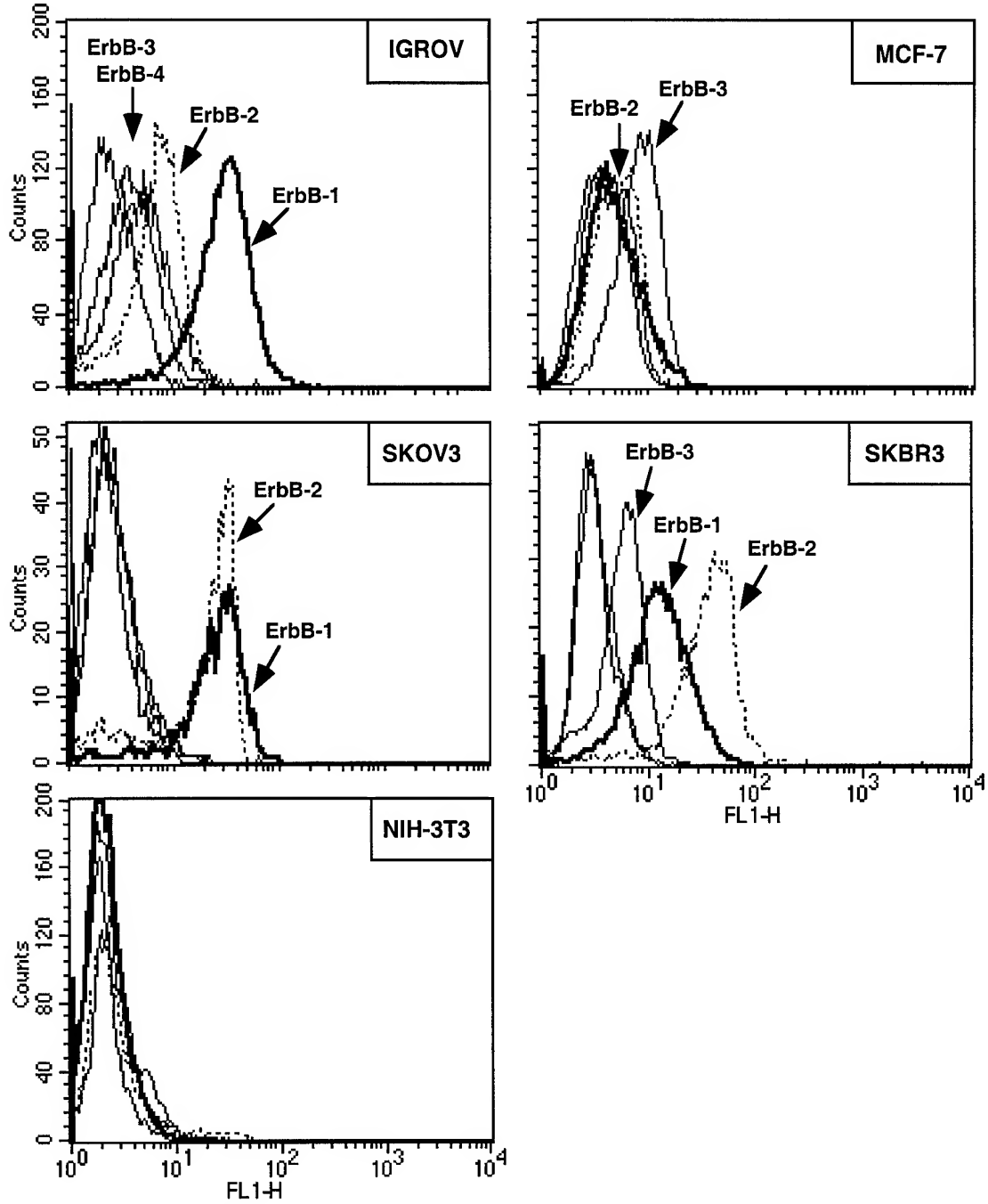


Figure 24. FACS analyses of tumor cell lines stained with anti-ErbB-1, ErbB-2, ErbB-3, ErbB-4, and control antibodies.

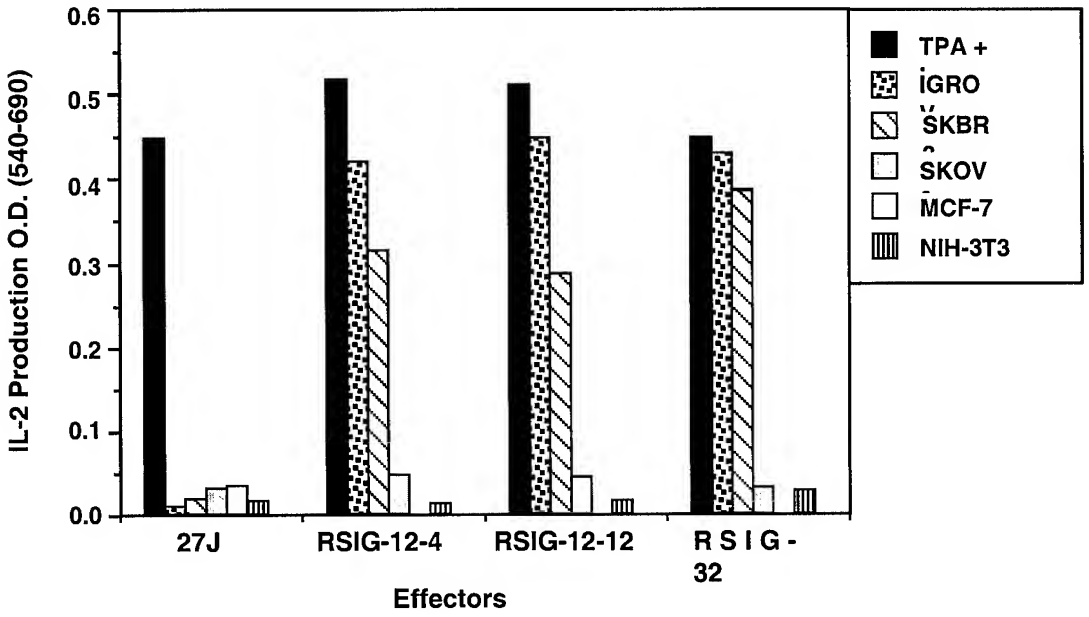


Figure 25. Interleukin-2 production by neuregulin-gamma transfected T lymphocytes stimulated by tumor cell targets. Supernatants were collected and tested for their ability to support the proliferation of an IL-2 dependent CTL-L line using the methyltetrazolium colorimetric assay.

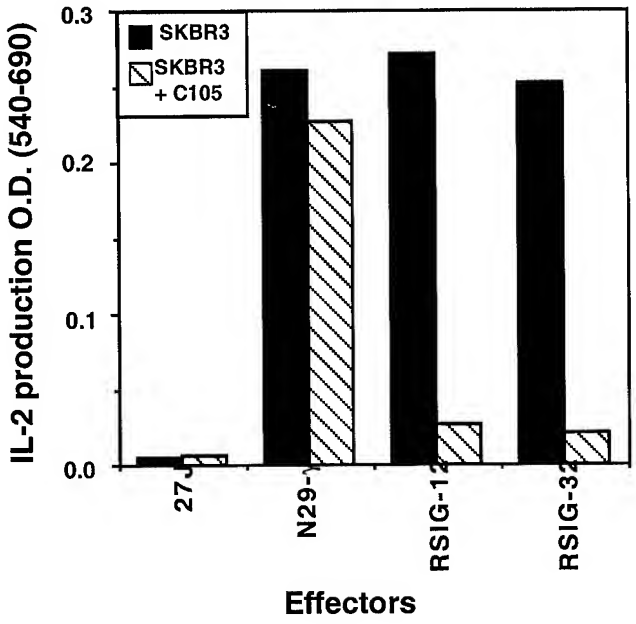


Figure 26. Anti-ErbB-3 antibody (C105) inhibits interleukin-2 production induced by SKBR3 stimulation of neuregulin (RSIG) but not anti-ErbB-2 (N29-γ) chimeric receptor expressing transfectants.

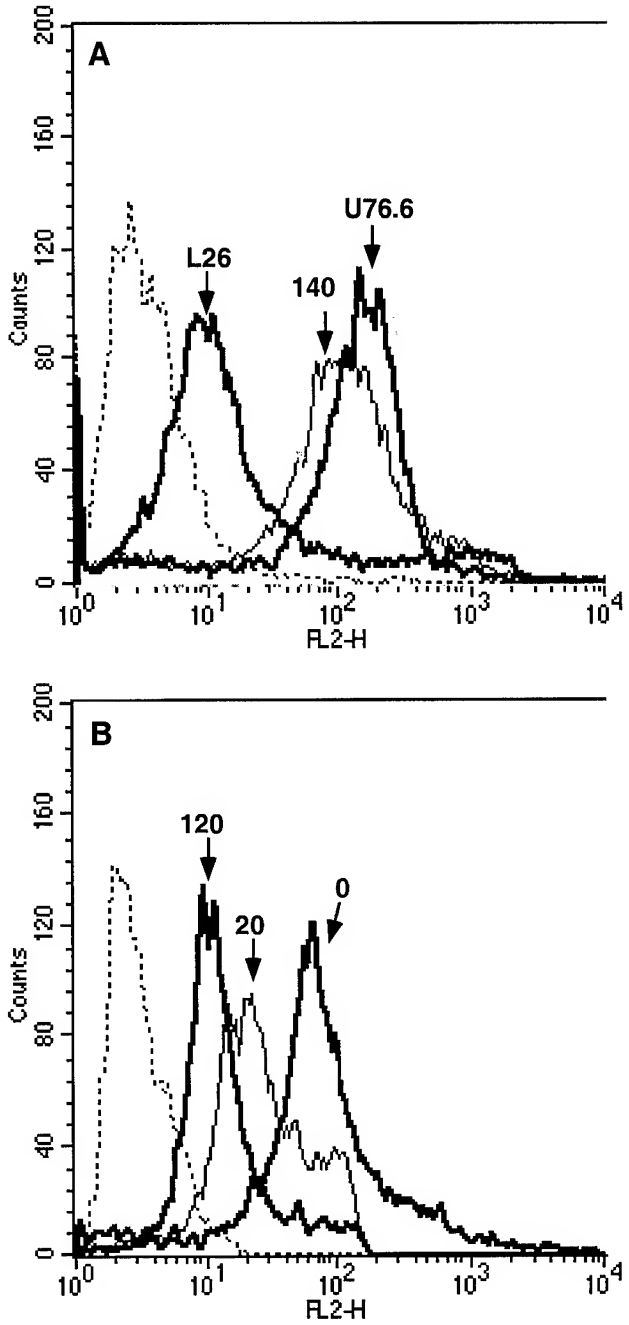


Figure 27. (**A**) Internalization of ErbB-2 receptors mediated by anti-ErbB-2 monoclonal antibodies. Antibodies were incubated with SKBR3 cells on ice in order to adhere and then antibody bound cells were incubated at 37°C for 90 minutes to allow receptor internalization. ErbB-2 receptor levels were determined by labeling with biotinylated anti-ErbB-2 (N29) antibody staining, detected by PE-strepatavidin and analyzed by FACS. Shown are the internalization effects of L140, L26, or isotype matched control anti-DNP antibody U7.6. (**B**) Kinetics of anti-ErbB-2 antibody-mediated internalization of ErbB-2 receptor on SKBR3 cells. Anti-ErbB-2 L26 monoclonal antibody was reacted with SKBR3 cells on ice followed by receptor internalization at 37°C for 0 minutes, 20 minutes, or 2 hours. Isotype-matched anti-DNP antibody U7.6 was incubated with SKBR3 cells on ice followed by 2 hours at 37°C as a control. ErbB-2 receptor levels were determined by labeling with biotinylated anti-ErbB-2 N29 antibody staining, detected by PE-strepatavidin and analyzed by FACS.

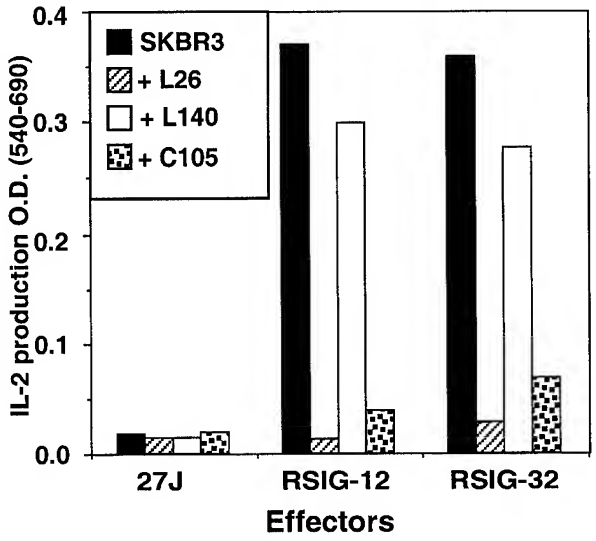


Figure 28. Inhibition of interleukin-2 production by neuregulin-γ transfected T lymphocytes stimulated by SKBR3 cells, in the presence of anti-HER2 (L140 and L26) and anti-HER3 (C105) antibodies.

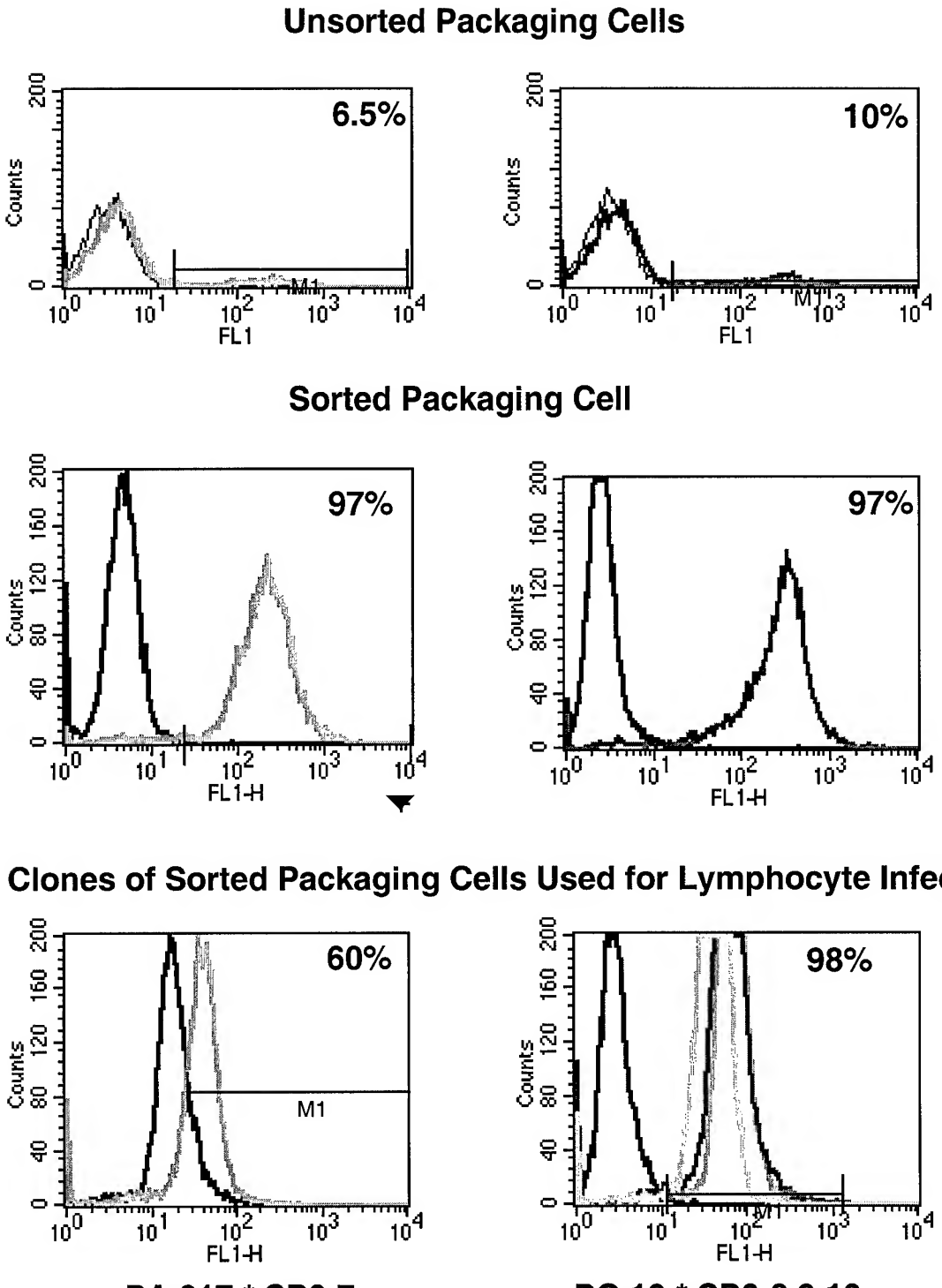


Figure 29- FACS analysis of packaging cell line construction. The left-hand side of the figure is the construction of the PA317-derived cell line that produces virus with an amphotropic host range. The right side of the figure is the construction of the PG13-derived cell line that produces virus with the gibbon ape leukemia virus envelope. The top figures are the results after the initial infection from the 'Ping-Pong' produced virus. For the infection of PA317, 6.5% of the cells expressed GFP and for the infection of PG13 10% produced GFP. The initial infected cells were sorted for fluorescence giving a population that was 97% positive for GFP in both infections. Clones from these infections were expanded and the ones from PG13 were very positive and the ones from PA317 were less so.

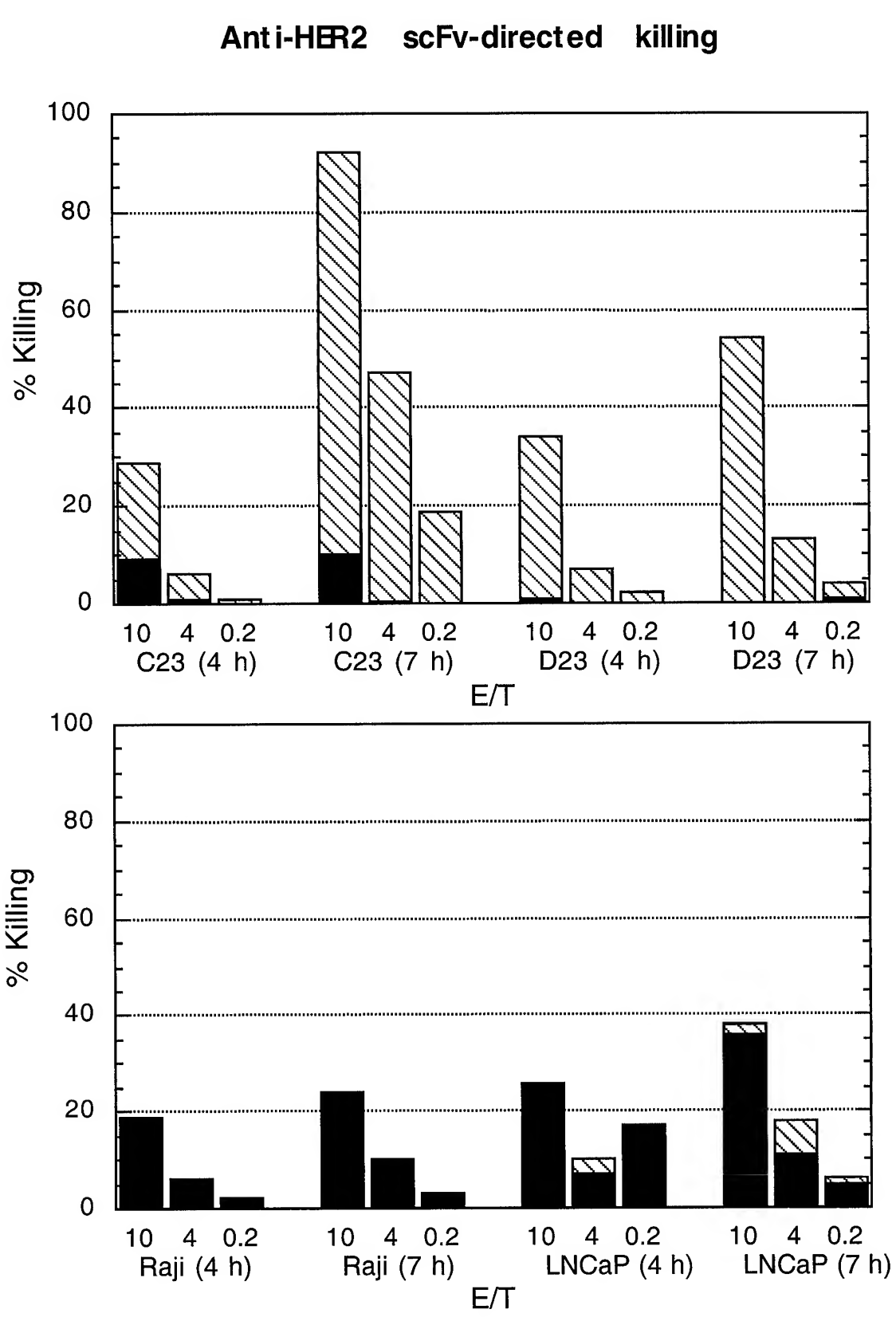


Figure 30- N29-directed killing by transduced human PBL. PBL were transduced with a chimeric receptor bearing an anti-HER2 scFv. Killing is measured by the release of ^{51}Cr. C23 is CHO transfected with HER2 and HER3. D23 is 32D transfected with HER2 and HER3. The solid part of the bar is the amount of killing done by uninfected lymphocytes whereas the striped bar is that done by transduced lymphocytes. The results for 2 different time points for each experiment are shown, 4 hours (4 h) and 7 hours (7 h).

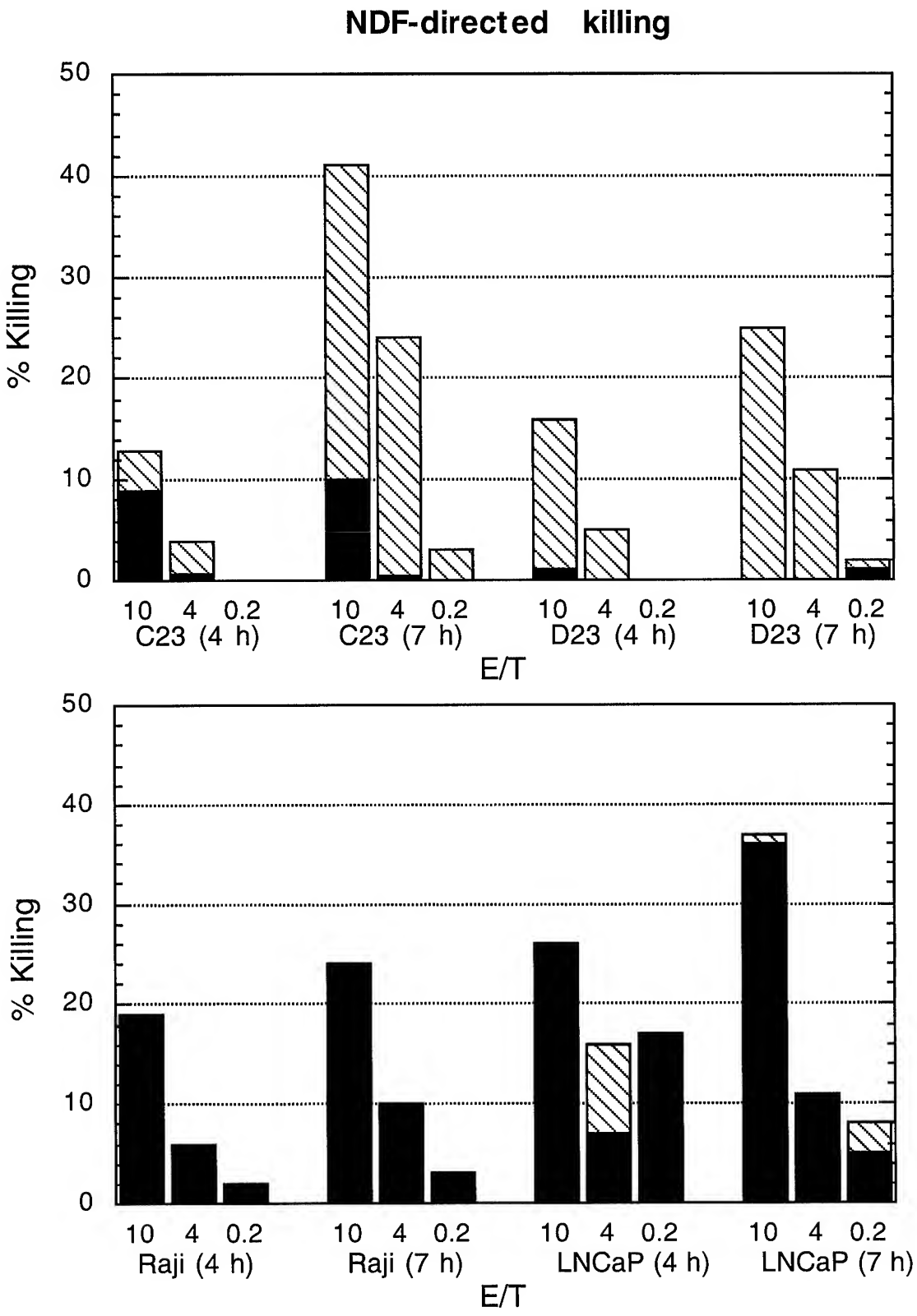


Figure 31- NDF-directed killing by transduced human PBL. PBL were transduced with a chimeric receptor bearing NDF. Killing is measured by the release of ^{51}Cr. C23 is CHO transfected with HER2 and HER3. D23 is 32D transfected with HER2 and HER3. The solid part of the bar is the amount of killing done by uninfected lymphocytes whereas the striped bar is that done by transduced lymphocytes. The results for 2 different time points for each experiment are shown, 4 hours (4 h) and 7 hours (7 h).

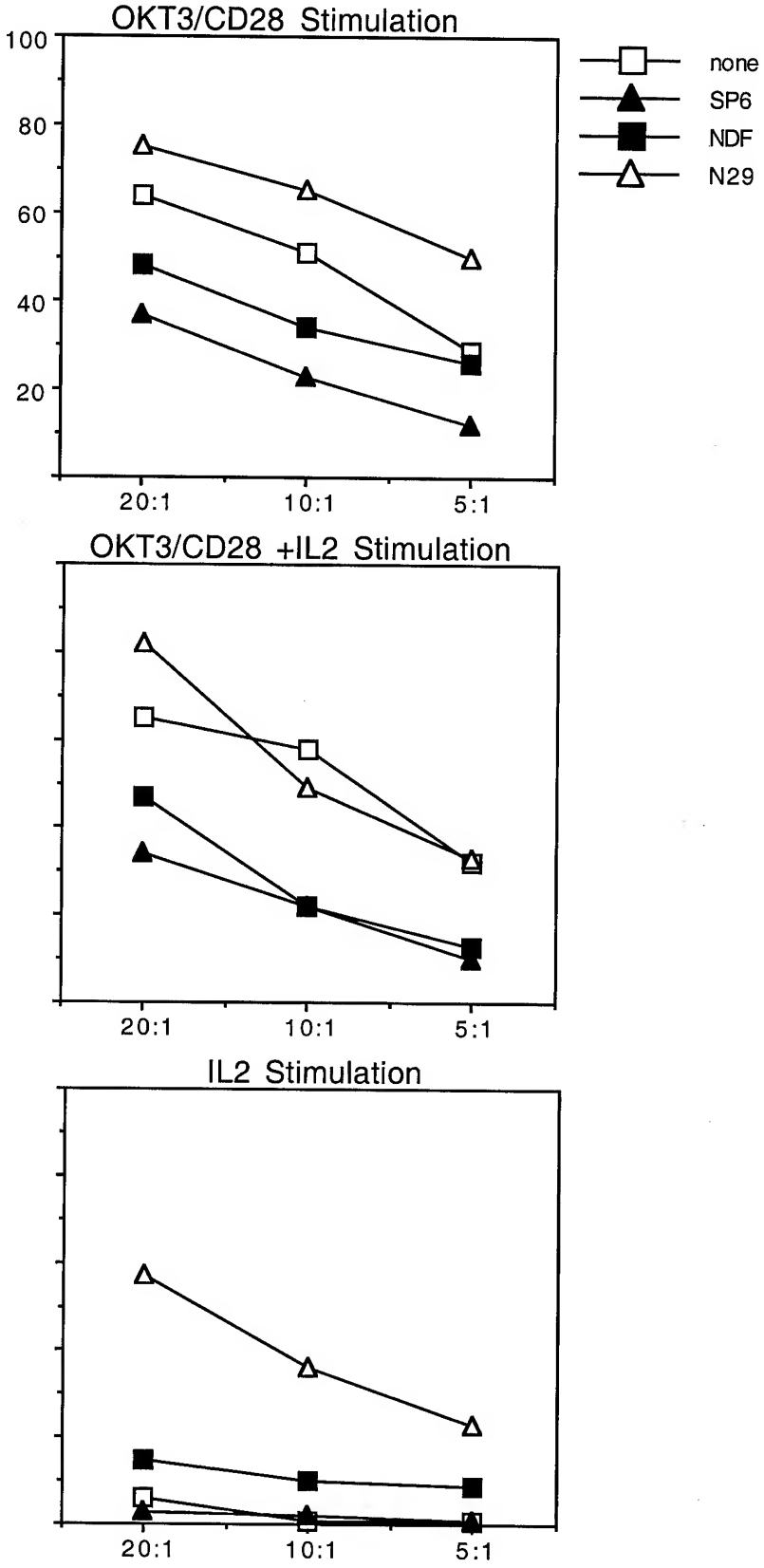


Figure 32. Killing of D23 target cells by transduced lympocytes. Lymphocytes were activated with different regimes before killing assay as described in previous figure.

Table I. Summary of HER2 receptor surface expression of tumor cells and their ability to stimulate T lymphocyte lines transfected with chimeric neuregulin-γ receptors.

	Degree of staining of:				Stimulation of
	HER1	HER2	HER3	HER4	transfectants
IGROV	++++	++/+++	+	+/++	+
MCF-7	--	+	++	--	--
SKOV3	++++	++++	--	--	--
SKBR3	+++	++++	++	--	+
NIH-3T3	--	--	--	--	--

6) KEY RESEARCH ACCOMPLISHMENTS

- Construction and demonstration of functionality of a tripartite T cell receptor composed of scFv, CD28 and FcεRγ
- Construction of a transgenic mouse containing and functionally expressing the gene for a tripartite scFv-anti-HER2-CD28-FcεRγ
- Construction of a tripartite T cell receptor using heregulin as the recognition unit to target effector T cells to HER2 expressing breast tumors
- Production of a retrovirus which can transduce anti-HER2 targeting agent tripartite receptors.

7) REPORTABLE OUTCOMES

Partial fulfillment of Ph.D. requirement for Dr Sara Feigelson

Partial fulfillment of Ph.D. requirement of Alain Ben-David

Acquired Cell Factory Grant from European Union

8) CONCLUSIONS:

In this reporting period we have been studying the construction of tripartite receptors consisting of a targeting molecule such as a scFv or a ligand, a portion of the co-stimulatory molecule CD28, and the signaling portion of the cytoplasmic domain of the Fcε receptor γ chain. We have expressed these molecules in the splenocytes of transgenic mice and human lymphocytes which have been transduced with this gene. These chimeric receptors appeared functional and demonstrated a full T cell stimulation upon encountering the target antigen. We strongly believe that this receptor configuration is more advantageous than the previous one we and others were studying. In the next period we will establish the function such as tumor killing of these modified lymphocytes in in-vivo models.

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